CELL PERMEABILITY STUDIES IN ISOLATED ORGANS

An Investigation of the Sugar Transport Process in Cardiac Muscle Cells and of the Action of insulin on this Process

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ABSTRACT

The work to be described was undertaken in order to substantiate and extend the work of Fisher and Zachariah (1961) on the action of insulin on the penetration of non-metabolised pentoses into the cells of the perfused rat heart. These workers found that sugar uptake by this preparation conformed best to kinetics based on the carrier transport hypothesis of Widdas (1954), whereby the sugar is assumed to be transported across the cell membrane in combination with a carrier molecule and released into the cell. This process is illustrated below.

Cell Membrane

$$\underset{\text{(outside)}}{\text{Sugar}} + \text{Carrier} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Complex} \overset{k_3}{\rightleftharpoons} \text{Complex} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{Carrier} + \underset{\text{(inside)}}{\text{Sugar}}$$

k_1, k_2 and k_3 are rate constants of reactions involved in the transport process.

From this model of transport, the following equation was derived (modified from Fisher and Zachariah, 1961).

$$(K + x)^2 \ln \frac{1}{1 - f} - x (K + x) f = KVt \quad \ldots\ldots (1)$$

x = the concentration of sugar outside the cells

f = the concentration of sugar inside the cells expressed as a fraction of that outside

t = time

K = the apparent dissociation constant of the sugar-carrier complex $\frac{k_2 + k_3}{k_1}$

V = the constant of maximal transport = $\frac{Ck_3}{2}$ where

C = the total amount of carrier involved in transport.

Fisher and Zachariah used a known constant concentration of either xylose or arabinose in the medium perfusing the rat heart, and estimated values of f after different periods (t) of perfusion. They were then able to determine the values of K and V for each sugar which gave closest conformity of the results to the carrier hypothesis.

They found that in the absence of insulin the values of K for the sugars were very small indicating that the carrier molecule had a very high apparent affinity for the sugars. When a low, submaximal concentration of insulin was added to the perfusate, the values of K were increased indicating that insulin diminished the apparent affinities of the carrier for the sugars, but the values of V were decreased.

One might expect intuitively that insulin would accelerate sugar transport by increasing the affinity of the carrier for a sugar but Fisher and Zachariah were able to show, on the basis of the carrier model, how sugar transport would be accelerated when the apparent affinity of the carrier for the sugar was decreased and it was assumed that this occurred as a result of a combination of insulin with the carrier. They proposed a hypothesis of insulin action based on their findings and accounted for the diminution in V brought about by insulin by assuming that in the presence of insulin sugar transport would be mediated almost entirely by that fraction of the carrier which had been modified by combination with insulin, so that the total amount of carrier (C) involved in effective sugar transport would be smaller than that involved in transport when no insulin was present.

There were several gaps in the evidence produced by Fisher and Zachariah in support of the carrier mechanism of sugar transport and of their suggestion as to how insulin might affect this

mechanism.

(i) It is a property of equation (1) that the higher the sugar concentration outside the cells, so the lower should be the value of f at any given time. Fisher and Zachariah used only one sugar concentration throughout their experiments, so that this test of the equation remains to be made.

(ii) These workers used only one insulin concentration in their experiments. Their hypothesis predicts that if the insulin concentration is increased above the submaximal concentration which they used, then the value of K for any sugar should not alter further, but V should increase because the fraction of total carrier involved in effective transport (i.e. that fraction modified by insulin) should increase. The limiting value of V, at the insulin concentration having the greatest effect on sugar transport, should therefore be the same as the value found in the absence of insulin, since all the carrier should once again be involved in the transport process.

In addition to these gaps in the evidence, a disquieting feature of the work of Fisher and Zachariah was the variability of the sugar-uptake results, and there seemed to be two main reasons for this:-

(i) The analytical techniques involved in the sugar uptake measurements provided a source of variation which it was felt might be better controlled by using automatic estimation techniques.

(ii) Another source of variation lay in the response of the animals used in these experiments to variations in environmental factors from day to day, (Young, 1960). This should be eliminated by keeping the animals under conditions of constant temperature and by regulating the period of daily illumination by artificial means.

Two other major advances have been made in improving the techniques involved in this work. Firstly, the perfusion apparatus has been modified so that continuous filtration of the medium perfusing the heart is achieved by means of a hardened paper filter rather than the sintered glass filter used by Fisher and Zachariah. With the

paper filter present it has been found that hearts can be maintained on a modified Krebs bicarbonate medium containing pyruvate to act as nutrient without including in this medium the plasma protein which Fisher and Zachariah had found to be necessary to sustain the hearts in a state of normal contractility in their apparatus. Thus permeability is studied in the present work in the absence of the complicating influence of a plasma protein preparation of undetermined composition. Secondly, the technique of estimating K and V from the experimental data has been improved.

In the work to be described, therefore, the work of Fisher and Zachariah is repeated using considerably improved techniques. In addition, the predictions which were made on the basis of their hypothesis are tested thoroughly. The uptakes of xylose and arabinose by the perfused heart have been studied over a range of perfusate sugar concentrations both in the presence and absence of different insulin concentrations.

The results confirm the prediction that the transport process conforms to the carrier mechanism at all the sugar concentrations studied.

The findings of Fisher and Zachariah that the values of K for the pentoses are very small in the absence of insulin, and that the values are increased by insulin are also confirmed. As the insulin concentration of the perfusate is increased, the K values do not alter significantly and this is again in agreement with the prediction. However, as the insulin concentration is increased, the values of V for the sugars increase in approximate proportion, so that at high insulin concentrations they greatly exceed the value which is predicted to be the limiting one on the basis of the original hypothesis.

At first sight these findings suggest that insulin has two actions of permeability:-

(i) an all-or-none action in increasing K, and
(ii) a graded action in increasing V.

However, both K and V are functions of the rate constant k_3 of sugar-carrier complex movement through the membrane, (see equation (1)) and it will be shown that the two effects can be accounted for completely in terms of the effect of insulin on this rate constant.

The resultant hypothesis of insulin action can be described in simple physico-chemical terms as an effect of insulin on the tendency of the mobile carrier molecule to associate with fixed elements in the cell membrane.

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GENERAL INTRODUCTION

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As long ago as 1889 experiments had focussed attention upon the pancreas as a source of some profound influence upon carbohydrate metabolism in the animal body. In that year von Mering and Minkowsky accidentally discovered that excision of the pancreas gave rise in the animal to a condition similar to that of diabetes mellitus. In 1901 Opie recorded that the islet tissue of the pancreas frequently appeared abnormal in diabetic humans, and later, in 1909, de Meyer gave the name insulin to the material which the evidence suggested came from the pancreatic islets and which seemed necessary to prevent the onset of diabetes.

The first practical procedure for insulin extraction was that of Banting and Best in 1922, and it was soon shown that insulin could maintain the blood sugar concentration of the diabetic animal at a normal level and could also reduce the level in normal animals, (Banting, Best, Collip, Macleod and Noble, 1922). Hepburn and Latchford (1922) first introduced the isolated

tissue preparation as a means to study the action of the hormone, and they showed that the disappearance of glucose from a medium perfusing the isolated rabbit heart was greatly enhanced by the addition of insulin to the medium. Best, Dale, Hoet and Marks (1926) found that both glycogen synthesis and glucose oxidation were stimulated by insulin. It became apparent, on considering the various effects, that both anabolic and catabolic processes were influenced by the hormone, and this focussed attention on the early steps of glucose metabolism, common to both processes, as a site of insulin action. Further progress in this field had to await the elucidation of the biochemistry of glycogen synthesis and of glucose catabolism, and after the discovery of hexokinase by Meyerhoff in 1927, it became clear that the product of glucose phosphorylation was a precursor not only of glycogen but also of the products of glucose catabolism.

Cori (1946) therefore considered that the insulin-sensitive step was the hexokinase-catalysed conversion of glucose to glucose-6-phosphate. Although no activation of hexokinase by insulin could be demonstrated, Price, Cori and Colowick (1945) and Price, Slein, Colowick and Cori (1946) purported to show that insulin could remove the inhibitory effect on this transformation of an anterior pituitary factor. However, other workers (Stadie and Haugaard, 1949) failed to repeat these findings and the observation of Perlmutter and Greep (1948) that insulin stimulated the glucose-uptake of diaphragms from normal rats just as markedly as it stimulated the uptake by diaphragms excised from hypophysectomised rats is inconsistent with the conclusions of Price et al. (1945, 1946). The balance of evidence is now strongly against the Cori hypothesis.

The main alternative to the hexokinase hypothesis was that the transport of sugar into the cells of certain body tissues was accelerated by insulin (the permeability hypothesis). This

possibility was considered by Fisher (personal communication) in 1937, but insulin induced alterations in intracellular sugar concentrations were at that time difficult to determine in view of the lack of a reliable indicator of the extracellular space of tissues. Lundsgaard (1939) suggested that insulin affected some transport mechanism and added that the effect was intimately associated with the cell structure, requiring intact cells for its demonstration.

It was not, however, until 1949 that the permeability hypothesis was supported by substantial experimental evidence. In that year, Levine, Goldstein, Huddlestun and Klein investigated the distribution of galactose in the body water of dogs from which the liver had been removed so that galactose could not be significantly metabolised and from which the kidneys had been removed so that the excretion of the sugar was prevented. They found that the volume of distribution of the sugar was 45% of the body weight. However, when insulin was injected with the galactose, the volume of distribution increased to 70% of the body weight. Since the

blood galactose level became stationary after the period of equilibration, it seemed unlikely that any significant utilisation of sugar occurred in the preparation. This assumption was supported by experiments employing eviscerated rats, (Levine, Goldstein, Huddlestun and Klein, 1950) where analysis of the carcasses showed that all the administered sugar could be recovered. Since the volume of distribution of galactose was increased in the eviscerated dogs from a low value to one corresponding approximately to the total water of the preparation, Levine and his colleagues concluded that insulin acted by facilitating the entry into the cells of the sugar which had previously been excluded. Similar conclusions were reached by Wick and Drury (1953). They showed that negligible amounts of C^{14}-galactose were utilised by the eviscerated, nephrectomised rabbit, and further demonstrated that while insulin considerably increased the final volume of distribution of injected galactose in these animals, the effect was diminished if glucose was injected at the same time. The workers attributed the latter

observation to competition between glucose and galactose for entry into the tissue cells.

These experiments set the permeability hypothesis on a firm foundation, and gave rise to a new approach to the problem of insulin action. By using a non-metabolised sugar for their experiments, Levine et al. (1949) dissociated the processes of intracellular sugar utilisation from that of the transport of sugar into the cell. The difficulty of demonstrating an acceleration of sugar transport by insulin using the physiologically encountered sugar, glucose, arises from the extensive metabolism of this sugar. Any stimulation by insulin of the glucose uptake of a preparation might simply reflect the stimulation of some intracellular catalysis involved in glucose metabolism rather than the facilitation of sugar entry into the cells. This point was considered in the experiments of Fisher and Lindsay (1956). They used the isolated, perfused rat heart, and making measurements of the intracellular compartment by deducting the extracellular (sorbitol) space from the total tissue space, they found that insulin increased

the intracellular accumulation of glucose. If insulin were simply accelerating an enzyme-catalysed reaction within the cell, the intracellular glucose concentration would be expected to diminish. Thus the effect of insulin was attributable to a stimulation of glucose transport in these experiments. Another point is worthy of mention in connection with this work with the perfused heart. Fisher and Lindsay also showed that the heart could survive when there was galactose but no glucose present in the perfusate. Furthermore, the intracellular accumulation of galactose, the non-metabolised sugar, was accelerated by insulin in these circumstances. Thus, acceleration of the membrane transport of galactose by insulin was demonstrated in the absence of the complicating influence of perfusate glucose. Since competition for the transport process was demonstrated between glucose and galactose by Wick and Drury, such competition makes the results of these workers, and of Levine et al., on the galactose distribution rather more difficult to interpret, for glucose was always present in the blood stream of the animals used in these experiments.

In turning attention to the precise mechanism by which insulin exerted its effect on cell permeability, most workers have approached the problem by studying the kinetic properties of the transport system and the effect of the hormone on these properties. In view of the foregoing account, it will be appreciated that the use of glucose as a test sugar for accurate quantitative studies of transport problems is made difficult by its intracellular utilisation, and so, adopting the approach introduced by Levine et al. (1949), workers have generally used non-metabolised sugars for such studies. It must be remembered that an effect of insulin on the transport of a non-metabolised sugar is only relevant to the problem of glucose metabolism if the particular transport mechanism is common to both sugars. The competition in vivo between glucose and galactose demonstrated by Wick and Drury (1953) suggests that this is the case for glucose and galactose, and other workers (Fisher and Lindsay, 1956; Battaglia and Randle, 1959; Park, Reinwein, Henderson, Cadenas and Morgan 1959) have repeated this observation and further

shown that several non-metabolised pentoses compete with glucose.

In considering the techniques available for studying the kinetics of sugar transport, it will be realised that there are serious objections to the use of intact or almost intact animals. The many tissues involved makes these preparations too complex, and it is also difficult to maintain a constant concentration outside the tissue cells of the sugar being studied, a condition necessary for accurate rate measurements. Again, the transport process for one sugar cannot be accurately evaluated when the circulation of the animal contains others which are likely to interfere with its transport. Attention was therefore directed at studying the process in isolated organs, but here again the choice of preparation requires careful consideration.

The isolated rat diaphragm is one test tissue which has attracted a great deal of attention since its sensitivity to insulin was first demonstrated by Gemmill in 1940, Kipnis and Cori (1957) and Helmreich and Cori (1957) used the rat diaphragm for their sugar transport

experiments, and it was employed in two forms. In the intact diaphragm preparation, the diaphragm itself was used, together with the rib cage, so that the muscle fibres which have their origins on the ribs remained intact. The transport of sugar into the muscle cells was studied by incubating the preparation in a sugar-containing medium for specific times. However, the removal of the muscle from its normal circulation established for the sugar a considerable diffusion path before the restrictive properties of the cell membrane are encountered. Thus, although Kipnis and Cori (1957) found that thiosulphate equilibrated almost completely with the extracellular fluid of the intact diaphragm within 5 min., Norman, Menozzi, Reid, Lester and Hechter (1959) found that sucrose required 15 min. If the transport of sugar into the muscle cells is a rapid process, the movement of sugar through the extracellular space is likely to be the rate-limiting process in such a preparation, so that uptake rates do not reflect the membrane transport step. The other rat diaphragm preparation is the cut diaphragm. Here the muscle is

excised from the rib cage, and the cells of the muscle fibres are damaged by this procedure. If such a preparation is incubated with a medium containing potassium ferrocyanide, a compound excluded from the cells of the intact diaphragm, the ferrocyanide readily penetrates the cells through the cut edges (Menozzi, Norman, Polleri, Lester and Hechter, 1959). Such a preparation is clearly unsuitable for membrane transport studies.

Another isolated tissue preparation, the perfused rat heart, which was first introduced for sugar transport studies by Bleehen and Fisher, (1954) has neither of the defects mentioned for the diaphragm preparations. Since the heart is cannulated by the aorta for perfusion through the coronary circulation, any compounds present in the perfusate are carried to the muscle cells by the normal circulatory route, and the diffusion path will be small. No cut cells are present, and in addition, the rate and strength of beat of the heart, and the flow rate of perfusate through it, can be used as indicators of the condition of the preparation (Zachariah,

1961). The stimulation of glucose utilisation by insulin (Bleehen and Fisher, 1954) and the insulin-sensitive transport of galactose and glucose, as well as competition between these sugars (Fisher and Lindsay, 1956) have all been demonstrated with the perfused heart.

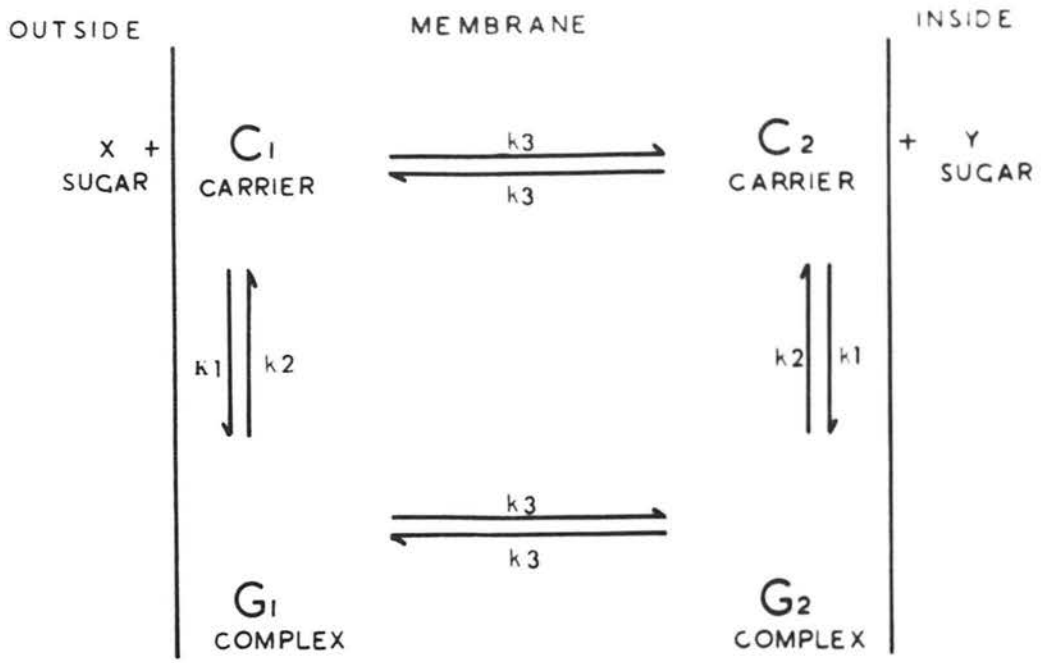
Zachariah (1961) performed extensive investigations of the suitability of the perfused rat heart for experiments making accurate quantitative assessment of the sugar permeation process. Experimental conditions were established such that the isolated heart could be maintained by perfusion for over two hours without significant change in the force of contraction. During this period, the permeability properties of the heart were found to change. There was a fall in the cell permeability over the first 30 min. of perfusion and evidence suggested that this was due to the loss of endogenous insulin activity. After this time, so long as dialysed ox serum was included in the perfusion medium, a period of stable permeability was established, suitable for quantitative studies on the sugar transport process. This period lasted for approximately

one hour and was followed by a rise in cell permeability and subsequently by heart failure. These findings are discussed in detail in Section III. It was the object of Fisher and Zachariah (1961) to use this test organ to study the time course of sugar uptake by the muscle cells and to examine the results for conformity to various kinetic schemes proposed for the transport process, for there was some disagreement in the literature as to the general nature of the process.

Kipnis and Cori (1957) using both the cut and intact diaphragm preparations, maintained that sugar uptake conformed to first order reaction kinetics, the uptake rate varying with the sugar-concentration of the incubation medium and Resnick and Hechter (1957) obtained similar results. In opposition to this work, an inverse relationship between the <u>in vivo</u> volume of distribution of a sugar and the amount of the sugar injected into the animal had been found by Wick and Drury (1953) in their experiments with eviscerated, nephrectomised rabbits, and Fisher and Lindsay (1956) found that the rate of uptake

FIG. 1.

MODEL OF CARRIER TRANSPORT MECHANISM*



x = concentration of sugar outside the cell

y = concentration of sugar inside the cell

C_1 and C_2 = concentrations of free carrier at the edges of the membrane

G_1 and G_2 = concentrations of the sugar-carrier complex at the edges of the membrane.

k_1 and k_2 = velocity constants of formation and dissociation of sugar-carrier complex. Their dimensions are 1/conc. x time and 1/time respectively.

k_3 = Transfer constant of free and combined carrier in the membrane. The dimension of k_3 is 1/time.

*Modified from Bowyer (1957)

of galactose by the cells of the perfused heart was not proportional to the galactose concentration of the perfusate. Fisher and Lindsay suggested that the lack of proportionality would be expected if sugar transport were mediated by combination of sugar with a specific carrier molecule in the cell membrane as described by Widdas (1954). Park, Reinwein, Henderson, Cadenas and Morgan (1959) found that the sugar uptake of the perfused heart was related to the perfusate concentration in a manner defined by Michaelis Menten kinetics and this supports the possibility that a carrier process might be implicated. Bowyer (1957) has concluded that most of the available work on non-electrolyte transport into erythrocytes is best accounted for by the mathematical treatment of Widdas (1952, 1954) based on the carrier model, (Fig. 1), and Fisher and Zachariah (1961) found that their results of sugar uptake by the isolated rat heart were best explained on the basis of this model both in the presence and absence of insulin. In this model, the following assumptions are made:

(i) Sugar traverses the membrane in combination with a membrane component, the carrier.

(ii) The carrier molecule moves freely within the membrane due to thermal agitation, and this movement is unaltered by combination with the sugar.

(iii) The rate of movement of the carrier can influence the equilibrium between the carrier and the sugar at the membrane surface.

In dealing with this carrier model of sugar transport, it is felt that the terminology used by Fisher and Zachariah (1961) is unsatisfactory. In what follows, therefore, the apparent dissociation constant of the sugar-carrier complex, which is taken as $\frac{k_2 + k_3}{k_1}$ rather than $\frac{k_2}{k_1}$ because of assumption (iii) above, will be referred to as K and not M. The total amount of carrier present is referred to as C, and the membrane constant, N used by Fisher and Zachariah is taken as the product KV by our terminology.

From the carrier model, the following equation

can be derived (modified from Fisher and Zachariah, 1961):-

$$(K + x)^2 \ln \frac{1}{1 - f} - x (K + x) f = KVt \ldots (1)$$

Where t = time

f = the intracellular sugar concentration expressed as a fraction of the external ($^{y}/_{x}$).

From equation (1) it will be seen that changes in t will produce proportionate changes in the left hand side of the equation so long as 'x' is fixed, for K and V are constants. Consequently the applicability of the carrier hypothesis can be tested by following the time course of penetration of sugars into heart muscle cells, and from the results, values can be estimated for the parameters of permeation, K and V, (Fisher and Zachariah, 1961). The values of K and V for different sugars can be determined and the changes in these parameters induced by the addition of insulin should throw light on the mode of action of the hormone on the transport process.

When the uptakes, by the perfused heart, of two non-metabolised pentoses, D-Xylose and L-Arabinose were studied in the presence and absence of insulin (Fisher and Zachariah, 1961) the following findings were made:-

(i) the kinetics of transport for both sugars, in the presence and absence of insulin, were consistent with the carrier hypothesis

(ii) the apparent affinity of the membrane carrier molecule for the pentoses was extremely high (K very small)

(iii) addition of a sub-maximal concentration of insulin increased the value of K for both sugars, and diminished the value of V.

Upon these findings the workers based a hypothesis of insulin action as follows:-

(i) it is assumed that insulin combines with the carrier molecule to form a complex with a lower affinity for the sugar (K increased by insulin) than that of the carrier alone.

(ii) the insulin-carrier complex is assumed to be more efficient at transporting the sugar, than is the carrier itself. The transport rate in the model will depend upon the concentration gradient of the sugar-carrier complex across the membrane. If the carrier is

equally distributed between outer and inner halves of the membrane, and if the fractional saturation of carrier by sugar is given by $\frac{x}{K + x}$ where K is the apparent ~~equilibrium~~ dissociation constant for the reaction, then the relationships shown in Table I can be derived (modified from the paper of Fisher and Zachariah, 1961). It will be seen that if K is very small, even at low internal sugar concentrations, sugar penetration is likely to be rapidly inhibited because of the early attainment of a high degree of saturation of carrier with sugar at the inner edge of the membrane. Increasing the value of K in these circumstances would decrease the fractional saturation of carrier at the inner edge to a much greater extent than that at the outer edge.

The concentration gradient of complex across the membrane would be increased and transport would be accelerated. This situation would be reversed if the value of K is increased above a certain value so that it lies in the region where a markedly reduced saturation of the carrier at the external boundary can occur. The values of

TABLE 1.

Effect of Alterations in the Equilibrium Constant of the Dissociation of Sugar-Carrier Complex on the Rate of Sugar Uptake

Apparent Equilibrium Constant (K) (mM.)	Conc. of Sugar-Carrier Complex		$xC_1 - yC_2$ (mM.)
	Outer Surface xC_1 (mM.)	Inner Surface yC_2 (mM.)	
0.1	0.9967	0.8571	0.1396
1.0	0.9677	0.3750	0.5927
10.0	0.7500	0.0566	0.6934
100.0	0.2308	0.0060	0.2248

Sugar concentration at outer surface, 30mM; sugar concentration at inner surface, 0.6mM. Total amount of carrier at each surface is assumed to be equal to 1mM. xC_1 = Fraction of available carrier combined with sugar at outer membrane surface; yC_2 = fraction of available carrier combined with sugar at inner membrane surface.

K found by Fisher and Zachariah for xylose and arabinose are very small, (0.21mM and 0.06mM resp.) and lie in the region where an increase in transport is brought about by increasing K, that is by diminishing the apparent affinity of carrier for sugar.

(iii) in the presence of insulin, since the insulin-modified carrier is more efficient at transporting sugar than the carrier alone then transport of sugar will be almost entirely by the modified carrier so that the total amount of carrier involved in transport will be less in the presence of insulin than in its absence. This point, which is difficult to establish in any simple fashion, is dealt with mathematically in Section I. Thus V, which equals $Ck_3/2$ diminishes when a sub-maximal concentration of insulin is added.

Scope of the present work

The foregoing work formed the background to the present investigation. Possibly the main feature of the hypothesis proposed by Fisher and Zachariah is that the acceleration of sugar transport by insulin is accounted for in terms of a reduction

apparent
in the/affinity of the carrier for the transported sugar, a proposition which is the reverse of what one might intuitively anticipate. The object of the present work was to substantiate this hypothesis by repeating and extending the observations made by Fisher and Zachariah.

The first section of this thesis is devoted to a mathematical exploration of the carrier model so that those aspects which readily lend themselves to experimental test can be established and the method by which to elucidate the metabolism of the sugar transport process and the action of insulin on this process clearly defined.

In the next two sections, attention is directed at making improvements in the techniques used in the work of Fisher and Zachariah. It was felt that the sugar uptake results exhibited a variability which might be diminished, thereby improving the reliability of the parameters calculated from these results. Two main sources of this variability were apparent. It was shown by Young (1960) that the insulin-dose response curve for sugar transport in the perfused rat heart was higher in summer than in winter, and

and the reason for this lay in the response of the experimental animals to variations in the period of illumination during the day at different times of the year. To eliminate variation from environmental factors, all animals for the present investigation were kept in conditions of constant temperature and exposed to alternating 12 hr. periods of darkness and light for at least 3 weeks prior to an experiments.

Another source of variation involved the analytical methods employed to estimate the pentoses (Roe and Rice, 1948) whose uptake was under investigation, and raffinose, (Cole, unpublished results) the compound used to measure the extracellular space of the heart. The methods were laborious, and with a large number of analyses, involved a certain amount of operator fatigue so that high accuracy was sometimes difficult to maintain. It was therefore felt that the use of automatic analytical procedures could improve the reliability of the results. The development of these is the subject of Section II of this thesis.

In the course of applying modified analytical techniques to extracts of the rat heart, it was discovered that serious difficulties accompanied the use of raffinose as an extracellular space marker. Therefore, in Section III an alternative method for measuring the extracellular space of the perfused heart is considered.

In the early perfusion experiments, the surprising finding was made that a heart could be permitted to survive for over 2 hr. on a medium lacking the dialysed ox serum which had been necessary in Zachariah's preparation, providing the perfusate filtration technique was modified. This discovery demanded that the permeability properties of the preparation be re-investigated before beginning kinetic studies and this investigation is described in Section III.

In addition to these technical considerations it was necessary, before performing the experiments to test the carrier hypothesis, to establish unequivocally the fraction of the total cell water in the heart muscle which is available for penetration by the pentoses. The different groups of workers studying the sugar transport

problem (Kipnis and Cori, 1957; Morgan, Henderson, Regen and Park, 1961) have made different assumptions concerning the volume of water within the cell into which the test sugar can penetrate in the presence and absence of insulin. Since the kinetics of sugar transport are conveniently studied in terms which include the determination of 'f', the fractional penetration of cell water by the sugar, and this is the approach used here, then the intracellular volume available for sugar entry forms the basis of all the sugar penetration measurements. In Section IV of this thesis the accessibility of the intracellular water to pentoses is explored both in the presence and absence of insulin, and the assumptions of other workers are examined in the light of the results.

The remainder of Section IV, and all of Section V, is devoted to studies on the sugar transport system in relation to the permeability hypothesis described earlier. It was felt that the work of Fisher and Zachariah could be extended profitably by testing the hypothesis of sugar transport and of insulin action on this transport process in two main ways. The first of these

concerns the relationship between the rate of transport of a sugar and its perfusate concentration. In the studies of Fisher and Zachariah (1961) the perfusate sugar concentration was always 30mM. It is a property of equation (1) (p. 16) which is clarified in Section I, that the relationship between the sugar penetration and its external concentration is one of inverse proportionality. This prediction which distinguishes a carrier mechanism from a diffusion mechanism is tested in Section IV.

Finally, only one insulin concentration was used in the experiments of Fisher and Zachariah, and this was a sub-maximal concentration (0.2mu. insulin/ml. perfusate). Consideration of the explanation postulated (p. 20) for the diminution in V ($-Ck_3/2$) by insulin makes it apparent that the addition of high concentrations of insulin should increase the fraction of total carrier (C) in combination with the hormone, thereby increasing V. At the concentration producing maximal stimulation of sugar transport, V should have returned to the value found in the absence of hormone, all the carrier being again involved

in the transport process, this time in combination with insulin. Furthermore, since it is assumed that in the presence of insulin the insulin-carrier complex acts as the effective sugar-transporting agent, then increase in the insulin concentration above the lowest level should not alter the value of K. Both of these predictions are tested in Section V.

In what follows, it will be seen that the relationship between sugar penetration and its concentration in the perfusate is as predicted by the carrier model at all concentrations studied. The constancy of K as the insulin concentration of the perfusate is increased is also found to be a valid prediction. On the other hand, as the perfusate insulin concentration is increased, V increases very much more than had been expected. This finding is readily explicable in terms of a simple extension of the original hypothesis.

SECTION I

THEORETICAL ASPECTS OF THE CARRIER MODEL

Theoretical Aspects of the Carrier Model

The assumptions implicit in the carrier hypothesis have already been set out in the General Introduction (p. 15). It is now necessary to explore thoroughly the carrier model mathematically, so that its properties might be clearly defined. Such treatment serves not only to indicate those aspects of the model which lend themselves to experimental test, but also to focus attention on the parameters characteristic of the sugar transport process. The action of insulin on the process can then be evaluated in terms of its effects on these parameters.

It is necessary, first, to account for the finding of several groups of workers (Fisher and Lindsay, 1956; Morgan and Park, 1958) that the rate of transport of sugars into the cells of the perfused heart fails to keep pace with increases in the concentration of sugar in the perfusion medium. In the model given in Fig. 1 p. 14 the speed of diffusion of the sugar carrier complex across the membrane is taken to be the rate limiting step, and this will depend upon the difference in the concentrations of the complex at the two edges of the membrane, thus -

Transport rate $\propto (G_1 - G_2) = k_3 (G_1 - G_2)$ where k_3 is the diffusion constant of the complex in the membrane.

But $G_1 = \frac{xC_1}{K + x}$ and $G_2 = \frac{yC_2}{K + y}$

Since the carrier is freely diffusible, at any instant, $C_1 = C_2 \ (= \frac{C}{2})$

Transport rate $\frac{dy}{dt} = \frac{Ck_3}{2} \left(\frac{x}{K + x} - \frac{y}{K + y}\right)$

When all the carrier is involved in transport, putting $\frac{Ck_3}{2} = V$, then $\frac{dy}{dt} = V \left(\frac{x}{K + x} - \frac{y}{K + y}\right)$

..... (2)

Thus $\frac{dy}{dt} = KV \left[\frac{x - y}{(K + x)(K + y)}\right]$

$$\frac{dy}{dt} = \frac{KV}{(K + x)} \cdot \frac{x - y}{(K + y)}$$

On integrating and rearranging this equation, we obtain -

$$-y + (K+x) \left[\ln x - \ln (x-y)\right] = \frac{KV}{(K + x)} \cdot t$$

Putting y/x equal to f, that is, expressing the sugar concentration inside the cell as a fraction f of the concentration outside, then we obtain the equation

$$(K+x)^2 \ln \frac{1}{1 - f} - x (K+x) f = KVt \ldots\ldots (1)$$

This is the form of the sugar transport equation used by Fisher and Zachariah. It is profitable at this point, however, to examine the equation further. Thus, for a fixed value of t and variable values of f and x, differentiating with respect to x we have

$$-(K + x)^2 \frac{-1}{1 - f} \cdot \frac{df}{dx} + \ln \frac{1}{1 - f} \; 2 (K + x)$$

$$- x(K+x) \cdot \frac{df}{dx} - (K+2x)f = 0$$

$$\frac{df}{dx}\left[\frac{(K+x)^2}{1-f} - x(K+x)\right] + \ln \frac{1}{1-f} \cdot 2(K+x) - (K+2x)f = 0$$

That is, $$\frac{df}{dx} = \frac{(K+2x)f - (2K+2x) \ln \frac{1}{1 - f}}{\frac{(K+x)^2}{1-f} - x (K+x)}$$

If the curve relating f to x shows a maximum or a minimum, then in either of these cases $\frac{df}{dx} = 0$

Thus, the condition for a maximum or a minimum is:

$$\ln \frac{1}{1 - f} \; (2K+2x) = f \; (K+2x)$$

i.e. $$\frac{\ln \frac{1}{1 - f}}{f} = \frac{K+2x}{2K+2x} = 1 - \frac{K}{2K+2x}$$

However, for all positive values of f $\ln \frac{1}{1 - f} > 1$

and for all positive values of x and K

$$\left[1 - \frac{K}{2K+2x}\right] < 1$$

Therefore, there is no circumstance in which K and x are positive in which there can be a maximum or minimum in the curve relating f to x.

Now, since $\frac{df}{dx} = \frac{f\ (K+2x) - \ln\frac{1}{1-f}\ (2K+2x)}{\left(\frac{K+x}{1-f}\right)^2 - x\ (K+x)}$

and since in all real circumstances f, x and K are positive, then it follows that f (K+2x) must be smaller than $\ln \frac{1}{1-f}$ (2K+2x) and that $\left[\left(\frac{K+x}{1-f}\right)^2 - x\left(K+x\right)\right]$ is positive.

It therefore follows that in these circumstances $\frac{df}{dx}$ is always negative.

From these considerations it may be concluded that f is a monotonically decreasing function of x. The inverse relationship between the rate of sugar uptake and the sugar concentration of the medium bathing the tissue is thus explained.

Use of the transport equation for kinetic studies

It has been mentioned that the equation used by Fisher and Zachariah was equation (1) given earlier. Using the results of their sugar uptake studies which yielded values for f and t, by substituting different values of K in the equation it was possible for these workers to

find an optimal value which gave the closest conformity of the sugar penetration results to the equation. This is an extremely tedious method and it was superseded in the present work by a more direct and precise method of determining the parameters of the sugar transport process from the equation. This method involves modification of equation (1) as follows.

We have $(K+x)^2 \ln \frac{1}{1-f} - x(K+x) f = KVt$

Therefore $\frac{f}{t} = \frac{K+x}{x} \frac{\ln \frac{1}{1-f}}{t} - \frac{KV}{x(K+x)}$

This equation will be referred to as the permeation equation.

It will be seen that so long as x is kept constant, since K and V are constants, then a plot of $\frac{f}{t}$ as the ordinate against $\ln \frac{1}{1-f}/t$ as the abscissa should give a straight line of slope $\frac{K+x}{x}$ and an intercept of $- \frac{KV}{x(K+x)}$. If a regression line computed from the results has a slope of b then

$$K = x (b - 1)$$

The intercept of the regression line, a is used to calculate V using:-

$$V = - \frac{abx}{b-1}$$

For conformity to the permeation equation therefore, the relation of $\frac{f}{t}$ to $\ln\frac{1}{1-f}/t$ must be linear, the slope of the line must be greater than unity, and the intercept on the $\frac{f}{t}$ axis must be negative.

A significant contribution of this technique is that it permits the quantitative assessment of the reliability of the K and V values calculated from any set of data. This is possible since the standard errors of the slope and intercept values of the regression line can be determined.

The osmotic correction

A further consideration in interpreting the values of K and V, derived as mentioned, is the net osmotic force to which the cells of the heart are subjected in a perfusion experiment. The medium perfusing the heart can be taken as being isotonic with the heart cell water (Conway and McCormack, 1953) before the test sugar is added (see Table 9). The addition of sugar to the perfusate should cause an osmotic withdrawal of water from the cells at the start of a perfusion.

If the sugar penetrates the cells, then the osmotic effect should diminish as sugar enters, but under such conditions, the time taken for the sugar to penetrate the cell water to a particular f value will be expected to be shorter than that theoretically derived when no osmotic effects are considered. This will be because the volume of intracellular water involved in the calculation of f should be smaller than its value in the absence of sugar in the external medium. Thus the values obtained for f at any particular time are likely to be erroneously high and the values of K and V finally computed will also be subject to error. The results of Section III support this argument. It will be demonstrated (p. 130) that the volume of intracellular water is dependent upon the sugar concentration of the medium perfusing the heart, smaller volumes accompanying the higher sugar concentrations at any particular perfusion time. It will be shown, too, that the cell water of hearts perfused with a sugar containing medium increases with the perfusion time, and that this effect is probably due to the entry of sugar into the heart cells.

These osmotic forces can be allowed for in the kinetic model of sugar transport studied here in the following way.

Consider a mass of cells containing one litre of intracellular water, which is in osmotic equilibrium with a large volume of extracellular fluid which is m milliosmolar. Let an amount of solute be added to the external medium in concentration x milliosmolar. Suppose that in a time t, y milliosmoles of solute enter the cells, and that this does not alter significantly the concentration of solute in the large volume outside the cells. The contribution of the extracellular indicator, which is less than 1% of the total osmolarity, is ignored here. Assuming that water movement is so rapid that there is osmotic equilibrium at all times, then the water content of the cells at the time t equals

$$\frac{m + y}{m + x} \text{ litres}$$

The intracellular sugar concentration will not now be y milliosmolar, but will be

$$y\left(\frac{m + x}{m + y}\right) \text{ milliosmolar}$$

Thus, at time t, the transport rate expression is not

$$\frac{dy}{dt} = \frac{KV}{(K+x)} \cdot \frac{(x-y)}{K + y} \quad \text{(see p. 28) but equals}$$

$$\frac{dy}{dt} = \frac{KV}{K+x} \cdot \left[\frac{x - y \frac{(m+x)}{(m+y)}}{y \frac{(m+x)}{(m+y)} + K} \right]$$

$$\text{Thus} \quad \frac{dy}{dt} = \frac{KV}{(K+x)} \cdot \left[\frac{x(m+y) - y(m+x)}{K(m+y) + y(m+x)} \right]$$

On rearranging and integrating this equation, we get

$$(m+x)(K+x) \ln \frac{1}{1-f} - (K+m+x) xf = \frac{mKV}{(K+x)} \cdot t$$

and therefore

$$\frac{f}{t} = \left(\frac{m+x}{K+m+x}\right) \frac{K+x}{x} \cdot \frac{\ln \frac{1}{1-f}}{t} - \left(\frac{m}{K+m+x}\right) \cdot \frac{KV}{x(K+x)} \text{*}$$

On comparing this osmotically corrected equation with the permeation equation (p. 31) it will be seen that the slope of the line obtained by plotting $\frac{f}{t}$ against $\ln \frac{1}{1-f}/t$ has ~~increased~~ altered by a factor of $\frac{m+x}{K+m+x}$ and the intercept has altered

* I am indebted to Professor Fisher for the derivation of this equation.

by a factor of $\frac{m}{K+m+x}$

From this it can be shown that the osmotically corrected parameters, Ko and Vo of the permeation process differ from the parameters K and V derived from the permeation equation in the manner shown below

$$Ko = K\left(\frac{m+x}{m+x-bx}\right) \qquad Vo = V\left(\frac{K+m+x}{m}\right)$$

Since m is of the order of 300 milliosmolar, x ranges from 10mM to 70mM, and b is never far from unity, then these expressions can be put in the approximate form

$$Ko \simeq K\left(1+\frac{x}{m}\right) \qquad Vo \simeq V\left(1+\frac{x}{m}\right)$$

The expression $\frac{x}{m}$ ranges from 0.03 to a value of 0.2, so that the osmotic correction will not be important unless x is large. In the final section of this thesis, a wide range of perfusate sugar concentrations will be employed so that consideration of the osmotic correction will be interesting.

Contribution of the fraction of carrier not modified by insulin to sugar transport

It is assumed by the hypothesis of insulin action proposed by Fisher and Zachariah that in the presence of insulin the total available carrier in the membrane will be functioning in two forms - (i) one fraction of the carrier will remain unmodified by insulin, so that its apparent affinity for sugar is unaltered. The parameters of transport by this carrier will be written as K_1 and V_1.

(ii) the other fraction of total carrier is assumed to be modified by insulin so that its apparent affinity for sugar is much lower than that of the unmodified carrier and the value of V is also assumed to be altered. The parameters for the insulin-modified carrier will be written as K_2 and V_2.

The net rate of transport in the presence of insulin, therefore, will be due to transport by both forms of the carrier so that we have

$$\frac{dy}{dt} = V_1 \left(\frac{x}{(K_1+x)} - \frac{y}{(K_1+y)} \right) + V_2 \left(\frac{x}{K_2+x} - \frac{y}{K_2+y} \right)$$

$$= \frac{K_1 V_1}{K_1+x} \frac{x-y}{K_1+y} + \frac{K_2 V_2}{K_2+x} \frac{x-y}{K_2+y} \quad \ldots\ldots\ldots (3)$$

Putting $p = \dfrac{V_1 K_1 K_2 (K_2+x) + V_2 K_1 K_2 (K_1+x)}{V_1 K_1 (K_2+x) + V_2 K_2 (K_1+x)}$

and $q = \dfrac{V_1 K_1 (K_2+x) + V_2 K_2 (K_1+x)}{(K_1+x)(K_2+x)}$

Then equation (3) becomes:-

$$\frac{(K_1+y)(K_2+y)}{(x-y)(p+y)} \cdot dy = q\,dt \quad \ldots\ldots\ldots\ldots \quad (4)$$

Putting $r = \dfrac{(K_1+x)(K_2+x)}{(p+x)}$ and $s = \dfrac{(K_1-p)(K_2-p)}{(p+x)}$

Then equation (4) becomes:-

$$\left(1 + \frac{r}{x-y} + \frac{s}{p+y}\right) \cdot dy = -q\,dt$$

This can be integrated to give

$$t = \frac{r}{q} \ln \frac{1}{1-f} - \frac{s}{q} \ln \left(1+\frac{x}{p}f\right) = \frac{x}{q} f^{*} \quad \ldots\ldots\ldots (5)$$

As an example of the nature of this relation, consider the situation in which the unmodified carrier has the parameters $K_1 = 0.048$, $V_1 = 2.22$ and the insulin-modified carrier has the parameters $K_2 = 1.2$, $V_2 = 1.2$. Let us put the

*I am indebted to Professor Fisher for the derivation of this equation.

external sugar concentration, x, as 30 mM. These values chosen for the parameters are similar to those to be presented later in the thesis which were determined experimentally.

In these circumstances, with a submaximal concentration of insulin present, it can be calculated that:-

$$\frac{r}{q} = 79.77640 \qquad \frac{x}{q} = 71.85120$$

$$\frac{s}{q} = -0.06622 \quad \text{and} \quad \frac{x}{p} = 73.64795$$

Taking the arbitrarily chosen values of f as:-

0.30, 0.35, 0.40, 0.45 and 0.50,

then the corresponding values of t can be determined from equation (5). From these values, it is possible to compute values of $\frac{f}{t}$ and $\ln \frac{1}{1-f/t}$ and to compute the regression line from these results in the manner to be used for the experimental data obtained later in the thesis. Then, using $K = x(b-1)$ and $V = -\frac{abx}{b-1}$ where b and a are the slope and intercept of the regression line (p. 31), it can be shown that

$$\underline{K = 1.25 \quad \text{and} \quad V = 1.28}$$

These values are experimentally not distinguishable from the values K = 1.2 and V = 1.2 for the insulin-modified carrier, with the errors of our experiments. A number of examples of this sort have been tried, and the answer is always the same. Thus, when insulin is present in the system, the values of K and V determined experimentally will be those of the insulin modified carrier, and in these circumstances, transport is affected almost entirely by that fraction of the carrier which has been modified by insulin. This conclusion is the basis of the assumption, therefore, that when a submaximal concentration of insulin is added the value of V should diminish, since the amount of total carrier involved in effective transport should be less ('C' diminishes).

SECTION II

THE AUTOANALYZER

CHAPTER I

Introduction

It has been mentioned in the General Introduction that the estimation techniques involved in the sugar-uptake studies of Fisher and Zachariah provided a source of variability which might be more stringently controlled. Two main types of compound require estimation; the pentoses whose uptake by the heart cells is under study, and the compound used to determine the extracellular space of the heart, (referred to as extracellular marker or indicator) raffinose. The techniques originally employed were those of Roe and Rice (1948) for pentoses and of Cole (unpublished method) for raffinose. Where the raffinose estimation is concerned, the reaction mixtures must be heated for 15 min. at 80°C. prior to cooling and measuring the optical densities. With the pentose estimation, after heating for 10 min. at 80°C. the mixtures must be allowed to stand in the dark a further 100 min. to attain maximal colour development. With each of these methods the timing of the addition of the various reagents is a decisive factor affecting the reproducibility of the results, so

that the methods become rather laborious procedures. For any one heart used in an experiment, the pentose and fructofuranoside must be determined, in duplicate, for both the heart and the perfusion medium. It was anticipated that our research programme would necessitate the use of a very large number of laboratory animals so that a great number of analyses would be involved. Clearly, the precision of these analyses will be a major factor affecting the reliability of the sugar-uptake measurements and accordingly it would be a great advantage if automatic estimation techniques could be developed.

An instrument already in use for routine clinical work in hospitals, the autoanalyzer (Technicon Instruments Corporation, New York) seemed a convenient one to permit such automation and this section is devoted to the use of this instrument. After describing the pieces of equipment comprising the autoanalyzer and listing some of the advantages and disadvantages of the machine, the modifications in reagents and techniques necessary in applying the original manual estimation methods to this machine are described.

Considerable difficulty was encountered in using the automatic technique to estimate raffinose in heart extracts, and this was overcome by using another fructofuranoside, inulin as the extracellular marker. At the end of the section a comparison is made of the variabilities exhibited by the manually and automatically performed analyses.

FIG. 2.

THE AUTOANALYZER

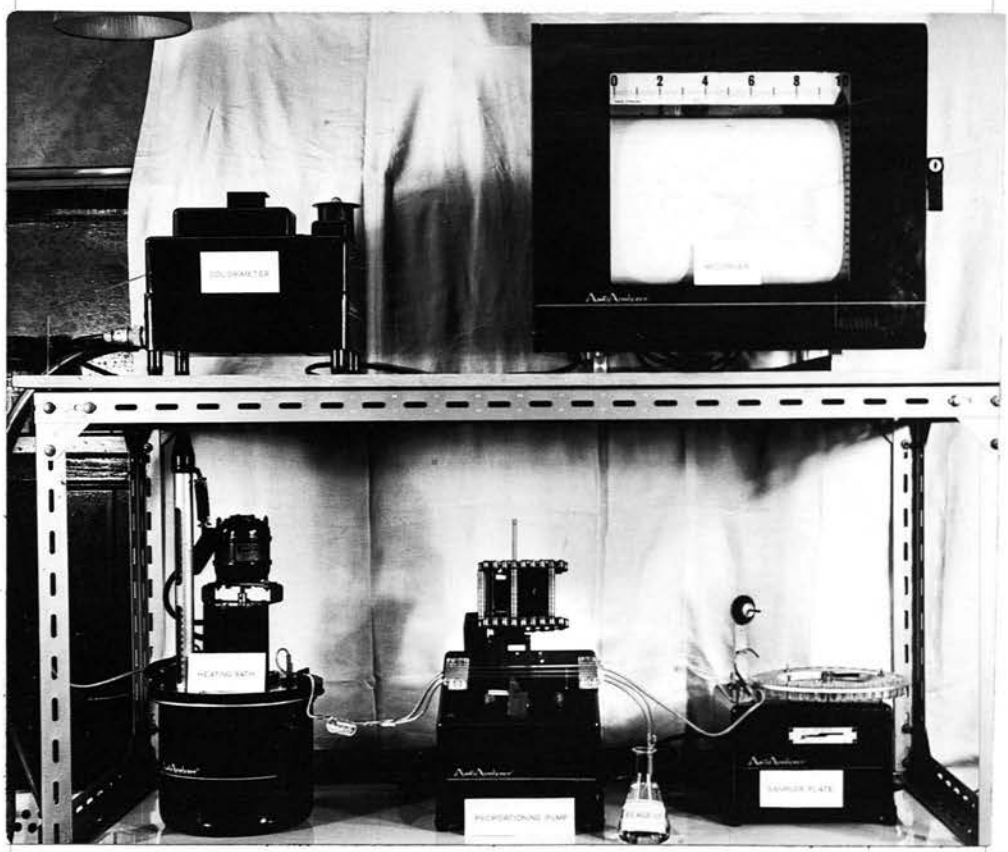
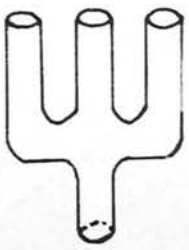
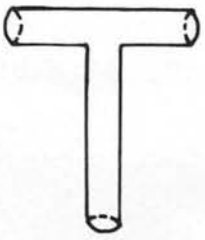


FIG. 3.

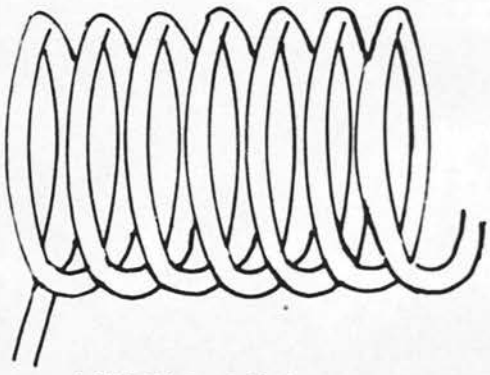


CACTUS



'T' TUBE

'H' TUBE



MIXING COIL

MISCELLANEOUS PIECES

CHAPTER II

Description of Autoanalyzer

The autoanalyzer consists of several separate pieces of equipment as follows:-

1. Sampler Plate
2. Proportioning Pump
3. Heating Bath
4. Colorimeter
5. Recorder

The entire autoanalyzer arrangement as it was usually used is shown in Fig. 2. The general principle of the system may be summed up in this way - aliquots to be analyzed are sampled, pumped along to mix with reagents in the requisite proportions, heated for a particular time at the specified temperature, colorimetrically estimated and a permanent record made of the results.

The main pieces of equipment are usually linked together in the desired sequence using flexible tygon tubing, of internal diameter two millimetres, to transport the liquid. Any divisions of the main stream require glass h-tubes, T-tubes or cactuses (Fig. 3). Small glass coils are used to ensure thorough mixing of solutions at any point.

1. Sampler Plate

This is essentially a platform, capable of carrying forty small cups of capacity 2.5 ml., which rotates at any one of three speeds. Attached at one side is a movable crook which carries the sampler tubing. As the platform rotates, so the sampler tubing dips into each cup in turn, an aliquot of liquid is withdrawn from the cup by the suction action of the proportioning pump, and it is passed on for mixing with the reagents.

2. Proportioning Pump

The pump consists of stainless steel cylindrical rollers which roll along a set of parallel, deformable plastic tubes carrying sample and reagents. The pressure of the rollers as they are driven over the tubes advances the liquids in the tubes. The volume of each liquid emerging depends only upon the bore of the particular tube, since the rollers travel at constant speed.

FIG. 4.

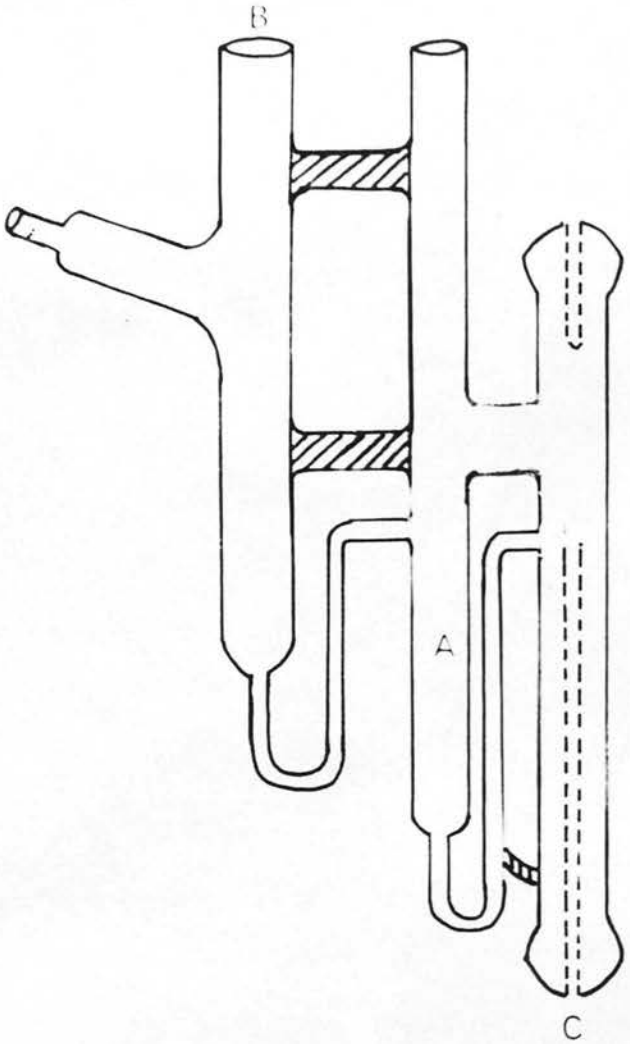


DIAGRAM OF FLOW CELL (Cuvette)

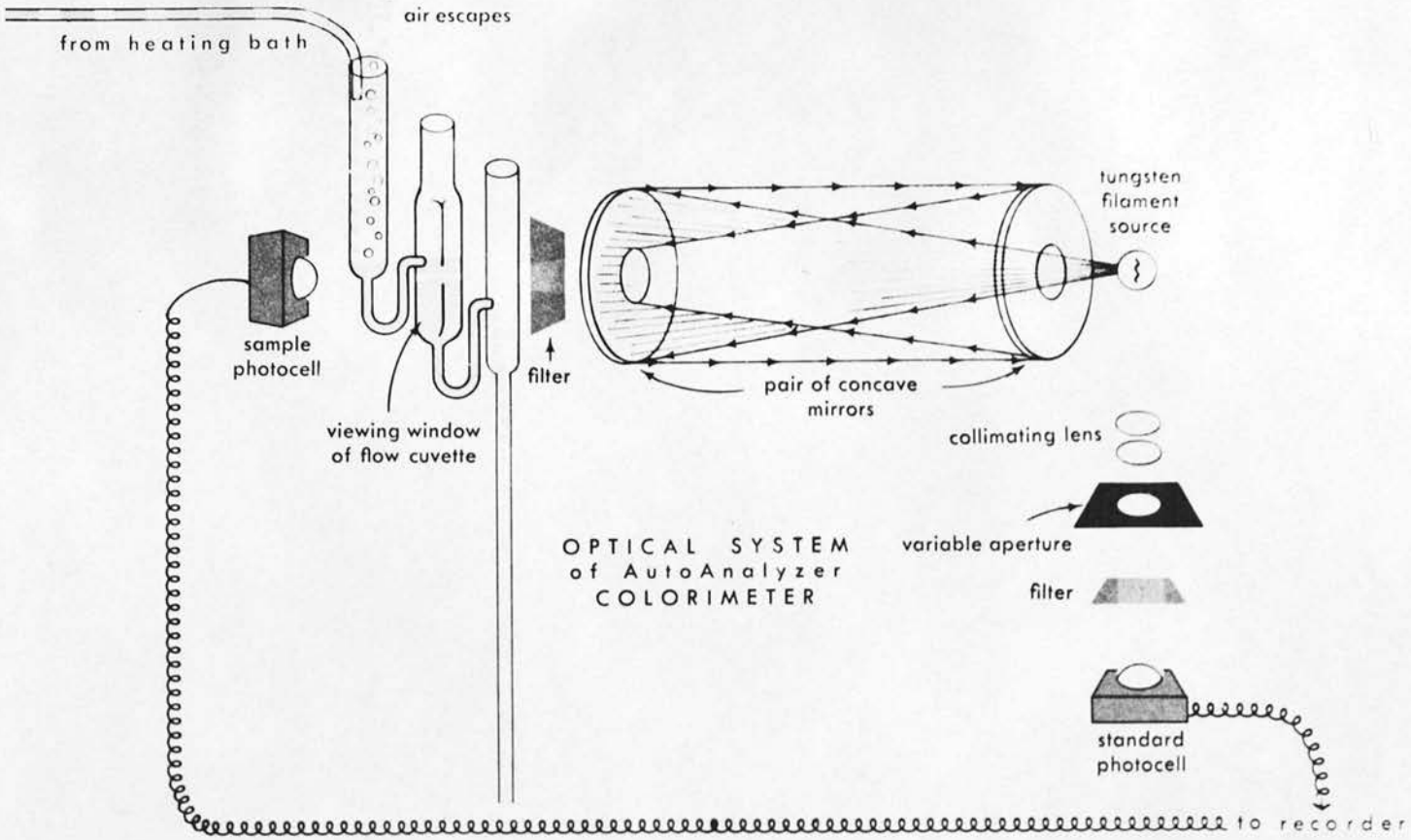
3. Heating Bath

Sample and reagents are thoroughly mixed prior to reaching the heating bath. The temperature to which the reaction mixture is heated can be varied from 20°C. to 100°C., whilst the time of heating can be varied from one minute to 30 min. for any one bath. This is achieved by passing the mixture through a helical glass tube of total volume 35 ml. immersed in a continuously stirred high boiling point liquid. The glass tube is rather like a large mixing coil as shown in Fig. 3. The heater of the system is controlled by a mercury contact thermoregulator, and temperature adjustment is by screw. The heating time for any reaction mixture is determined by the volume flow per minute of mixture through the bath, and this in turn is controlled by the bores of the tygon tubes selected for the proportioning of the reactants.

4. Colorimeter

The effluent from the heating bath passes into a glass cell of either 6 mm or 10 mm light path for colorimetric estimation - Fig. 4. The colorimeter beam is focussed upon the point 'A'

FIG. 5.



OPTICAL SYSTEM of AutoAnalyzer COLORIMETER

between the two dams of the cell. Any gas is allowed to escape from the system at 'B' the liquid itself emerging at 'C'. The optical system of the colorimeter is shown in Fig. 5. Two light beams are obtained from the same tungsten filament lamp and directed to fall on different photocells. The sample under estimation is interposed between the lamp and the sample photocell, and the difference in output between this and the second photocell, of standard output, is measured at the recorder. Identical interference filters of the desired wavelength are included in both sample and reference light paths.

5. Recorder

The instrument is a potentiometer recorder with a scale indicating the percentage transmission. In subsequent figures a scale of optical density is superimposed on the recorder scale.

Several general features of the autoanalyzer system merit attention here.

(1) Equal volumes of 'unknown' and standard solutions are treated identically throughout each procedure.

(2) When the pick-up crook is not aspirating

solution, air is sucked into the system and this serves to isolate the samples one from another.

(3) The colour yielding reaction need not proceed to completion since all mixtures are estimated at precisely the same stage of colour development.

(4) At the end of an analysis, the system is cleared simply by permitting the tubes to aspirate water in place of sample and reagents.

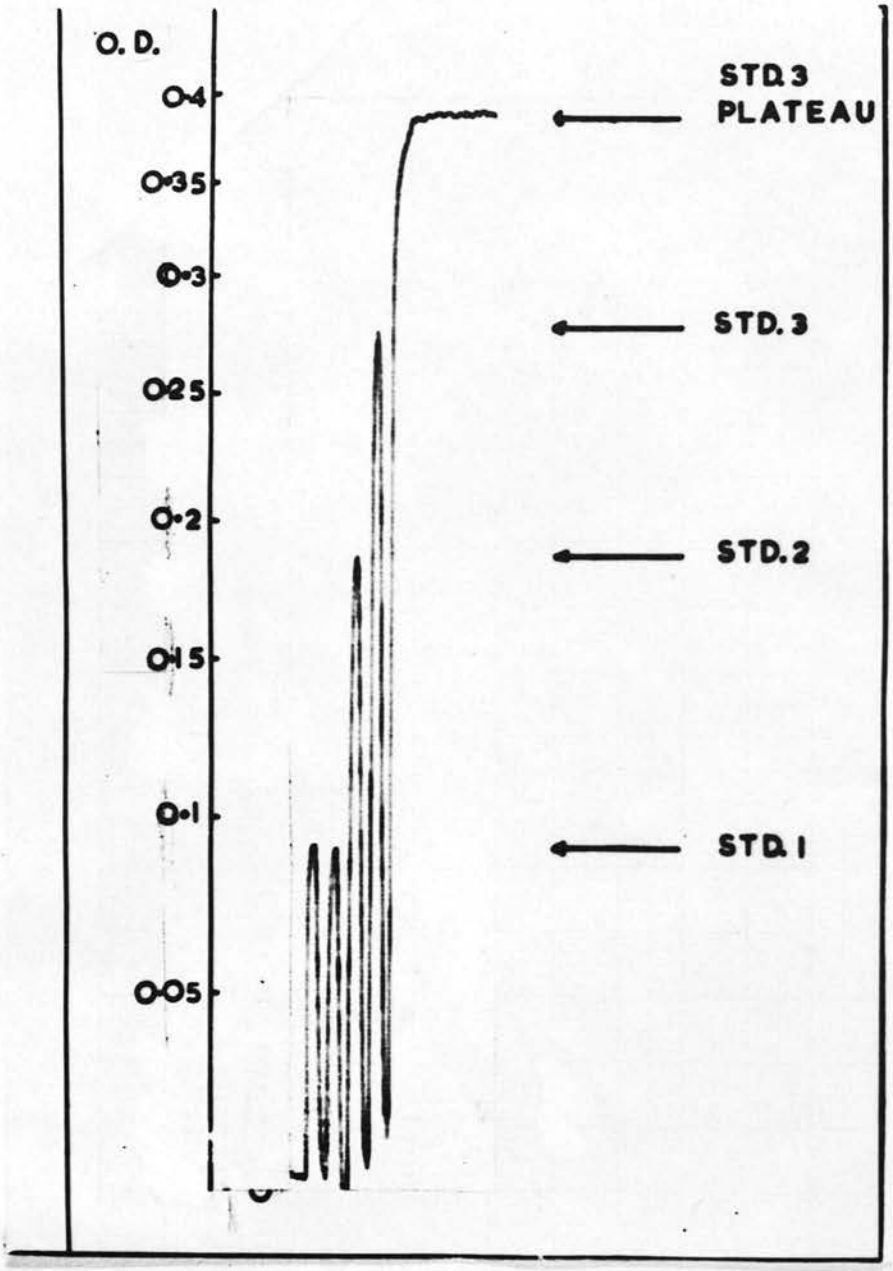
During preliminary experiments testing the value of the autoanalyzer in the present study, certain disadvantages became apparent and some basic rules for using the machine were evolved.

I. Because of the nature of the compressible tubes used in the pump, certain reagents have to be suitably diluted or avoided altogether, whilst the comparatively restricted time for colour development might require more concentrated reagents for best results. Manual analytical techniques may therefore have to be drastically modified for application to the automatic system.

II. The design of the flow cell (Fig. 4) is such that the volume of reaction mixture typically used in experiments is insufficient to displace com-

FIG. 6.

Peak heights for different sampling times



Standard 1,2 and 3 were run with the normal auto-analyzer sampling system operating, whilst the standard 3 plateau was obtained by continuously aspirating this standard along the sampler tube with the normal sampling system switched off.

pletely the liquid already lying in the cell. This results in dilution of the reaction colour as the mixture flows through the cuvette for estimation, and the peak height is diminished as illustrated in Fig. 6.

The plateau for the third standard was achieved by permitting an unusually large volume of reaction mixture to flow through the cell.

III. The **mixing of liquids of widely differing** refractive indices within the cuvette can cause the pen to move jerkily towards its next peak. This 'mixing' effect can be minimised by speeding up the flow through the system but in any case need not affect the final height of the peak. With each analytical technique an optimal flow rate is found permitting adequate colour development while the mixing effects remain at a tolerable level.

IV. The 'cleaning' action of air bubbles in isolating samples can be inadequate, and this is revealed both by the failure of the pen to return to a stable base-line between samples, and by discrepancies between several peaks given by the same standard solution as traces of residual material from the early mixtures contaminate the

later ones. This contamination can be lessened by including on the proportioning pump a tube of narrow bore which aspirates air, for this enhances the cleaning action. The defect can be finally overcome by interspersing cups containing water with the samples on the sampler plate. The wash-out of the flow cell between samples is then far more efficient, and although a stable base-line is not necessarily obtained, the peak heights accurately reflect the sample concentrations.

V. Occasionally a slight upward trend of the baseline could be detected when the machine was used for long periods. To counteract any possible errors from this effect, solutions are always symmetrically arranged as shown, where s represents a standard and x an unknown solution: water cups are represented by black dots.

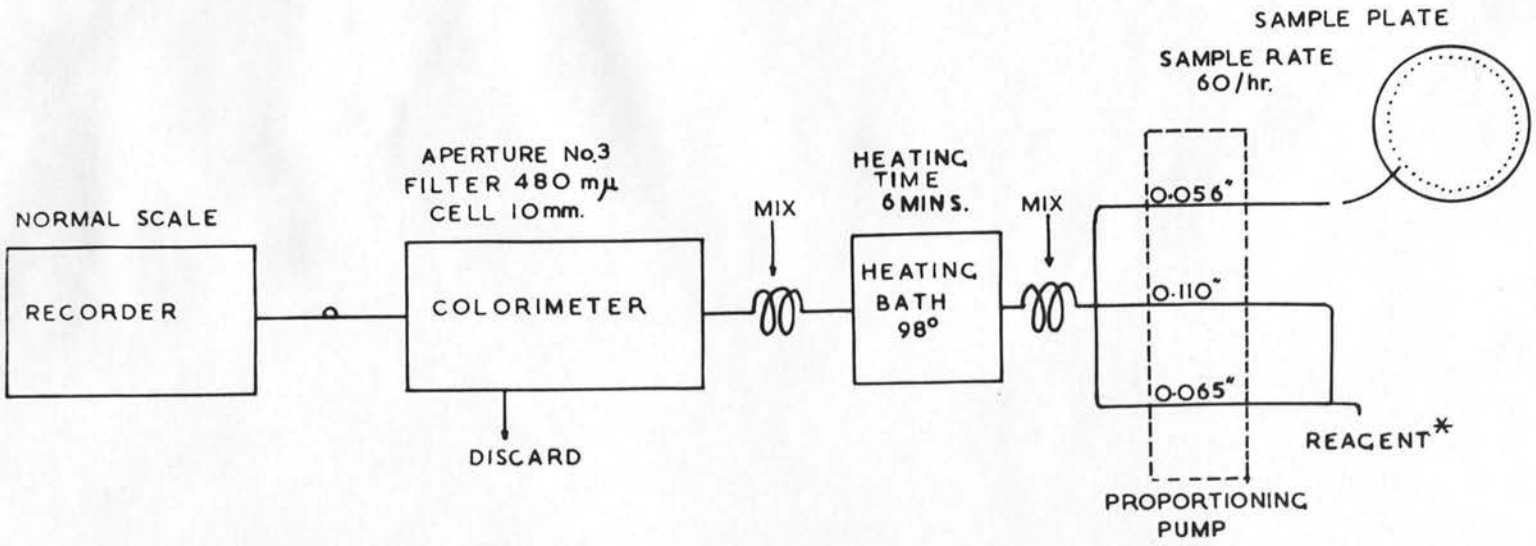
A

*

S_1 S_1 S_2 S_3 X_1 X_2 X_3 X_4 X_4 X_3 X_2 X_1 S_3 S_2 S_1 S_1

If the average values are used for calcuation of results, all the values are measured about the same mean point of the run (point 'A') and errors should be minimised for the upward drift was generally uniform throughout any run.

FIG. 7.



AUTOANALYZER SYSTEM – INULIN ESTIMATION (For Fructofuranosides)

*Reagent:- Concentrated hydrochloric acid containing 0.083 g. ferric chloride per litre diluted to 60% strength and containing 0.1% resorcinol finally.

CHAPTER III

The Estimation of Fructofuranosides and Pentoses

1. Fructofuranoside Estimation - Manual Technique

The method in its original form was developed by S.W. Cole (unpublished work) and employs the following reagents.

A - Resorcinal reagent:- 0.2% resorcinol in absolute ethanol.

B - Hydrochloric acid reagent:- concentrated hydrochloric acid containing 0.083 g. Ferric chloride per litre. Dilute one part with five of concentrated acid for use.

Two parts of A were mixed with three parts B and three parts of the solution to be estimated. After heating at 80°C. for 15 min., the optical density of the mixture was estimated in glass cells at 480 mμ.

Adaptation to the autoanalyzer and final procedure adopted

Before describing the modifications advisable in adapting the manual method to the instrument, the final procedure which was adopted on the auto-analyzer will be described. Fig. 7 shows the arrangement of the equipment for the estimation of

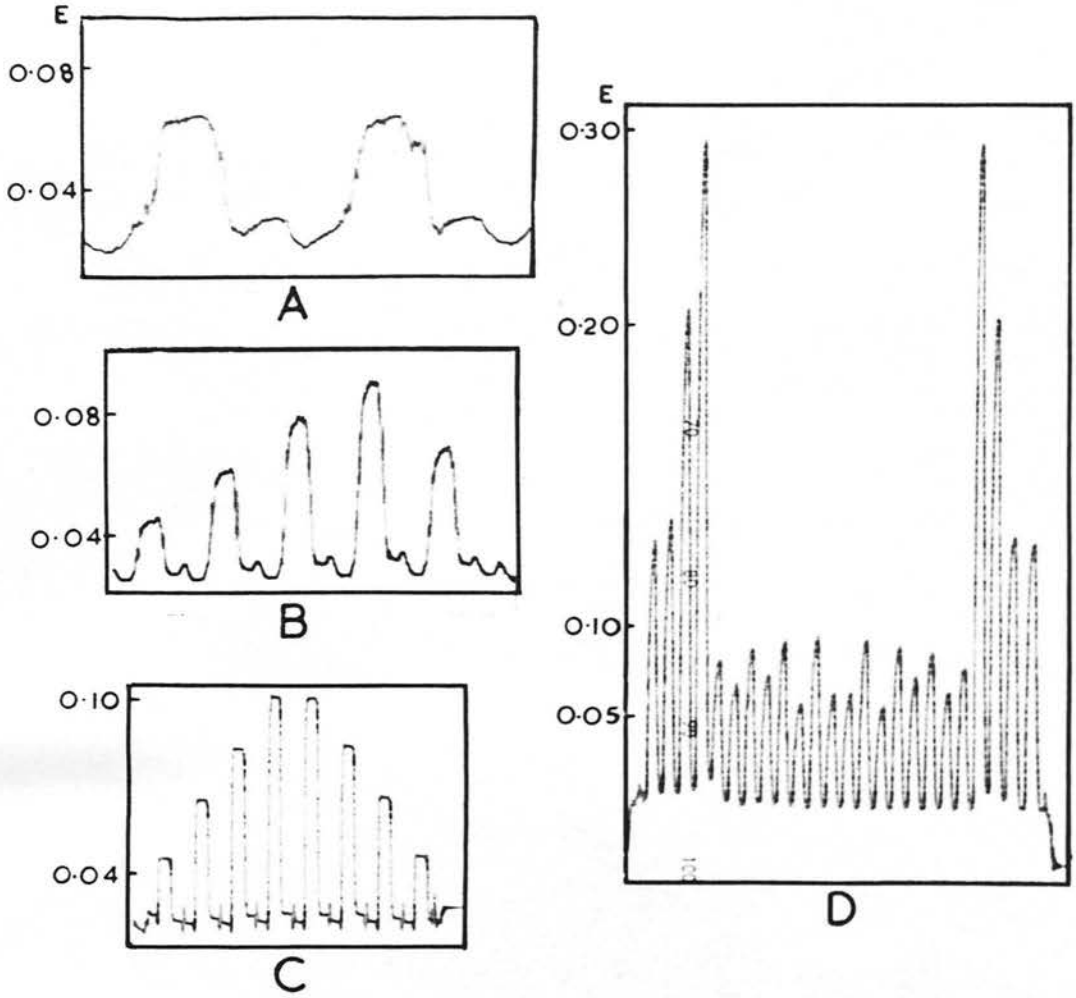
inulin but the method is equally suitable for raffinose, with which all the early experiments were made, and for other fructosans. Approximately 1.5 ml. of the samples to be analysed are placed in cups on the sampler plate, together with the requisite standard solutions (usually containing 15, 30 and 45 µg. of the compound under estimation per ml. of solution). The order of these solutions on the plate has been given on page 50 Water cups are interposed as described. The reagent tubes are inserted to the bottom of the reagent bottle, and with the sampler tubing in water, the proportioning pump started. After permitting the reagent and water to flow into the system for a few minutes, the sampler tubing is removed from the water and pushed through the crook on the sampler plate so that it dips to the bottom of the first cup. The analysis is begun immediately this operation has been completed by switching on the sampling device.

Modifications of manual technique

In the presence of concentrated hydrochloric acid the life of the autoanalyzer tubing was too short to be of value. The acid was therefore diluted, the final concentration adopted being

FIG. 8.

Raffinose Estimations



The conditions used for the Autoanalyzer runs giving the peaks of Fig. 8 are given below.

	A	B	C	D
Solutions used (µg. raffinose per ml.)	50	25, 50, 75 and 100, and one 'unknown' solution	25, 50, 75 and 100, then the same solutions in the reverse order	25, 25, 50 and 75 and 8 'unknown' solutions, then the reverse order
Sampling speed (samples/hr.)	20	20	40	60
Total flow rate(ml./min)	0.64	1.20	4.10	6.10
Proportion Reagent: sample	1:1	1:1	2:1	2:1
Cell light path(m.m.)	10	5	5	10
Heating time (min.) at 98°C.	38.5	23.1	8.1	6.1
Air* Segmentation	Yes	Yes	Yes	No

*This means of reducing contamination between samples has been mentioned on page 47

determined as described on page 56.

Alcohol attacked the tubes so that turbid solutions resulted, and these interfered with colour estimation. This was avoided by dissolving the resorcinol directly in the modified hydrochloric acid reagent. The determination of the best proportions of sample and reagent presented difficulties and this was largely accomplished by testing tubes of different internal diameters on the proportioning pump. The speed of sampling and the total flow rate of liquid through the system are also factors affecting the form of the peaks produced at the recorder, and these were varied in attempts to determine the most favourable conditions. A combination was found which gave high, clearly defined peaks for small amounts of fructosan. Fig. 8 illustrates some results obtained in early experiments with this estimation; in comparing the peak heights it should be noted that several conditions have been altered between runs.

Interference of pentoses with the fructofuranoside estimation

Preliminary experiments using a reagent con-

taining 1% resorcinol in 80% hydrochloric acid reagent, and a reaction temperature of 98°C. indicated that arabinose interfered drastically with an estimation of the raffinose content of a solution. This interference was investigated under different conditions of temperature, acid concentration and resorcinol concentration of the reagent.

Temperature and hydrochloric acid concentration

Solutions containing raffinose and arabinose in the concentrations indicated were prepared:-

R_1 25 µg. raffinose/ml. A_1 25 µg. arabinose/ml.

R_2 50 µg. raffinose/ml. R_2 50 µg. arabinose/ml.

These were run on the autoanalyzer in various final concentrations as shown

R_1 R_1 R_2 R_1A_1 R_2A_1 R_2A_2 A_1 A_2 then the reverse order.

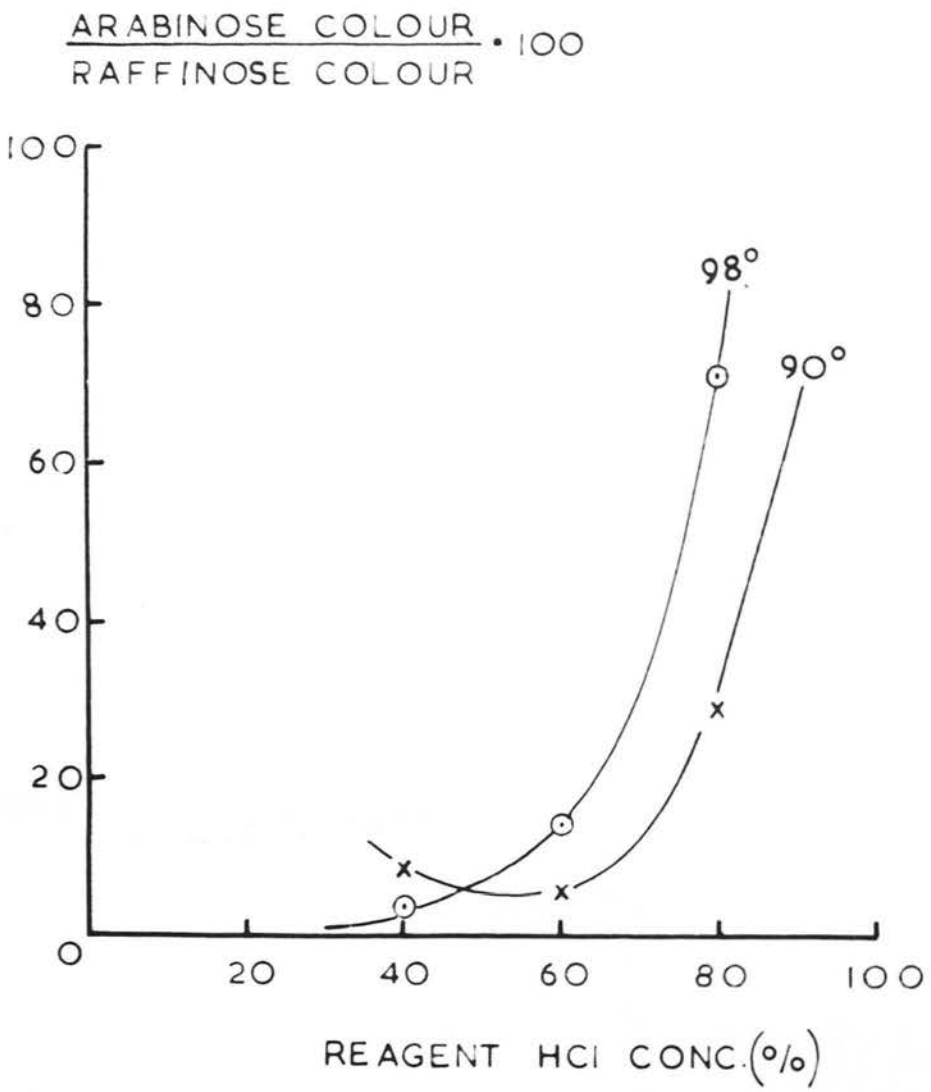
From the results a multiple regression was calculated based on the equation $Y = ax + bz + C$

where x = Raffinose concentration
z = Arabinose concentration
c = Blank value
Y = Predicted reading

It was therefore possible, from this regression, to

FIG. 9.

INTERFERENCE OF ARABINOSE WITH RAFFINOSE ESTIMATION PROCEDURES



The points have been calculated from the regression based on readings obtained on the autoanalyzer for mixtures of raffinose and arabinose as described in the text. They correspond to the values of $\frac{b}{a}$. 100 given in Table 2.

to compute the following values:-

'a' equals the increase in optical density per 25 µg./ml. increase in raffinose concentration (raffinose slope)

'b' equals the arabinose slope

$\frac{b}{a}$ 100 equals the arabinose colour as a percentage of that due to raffinose.

In addition, the standard errors of 'a' and 'b' could be calculated, (these were always equal in the present work) and also the residual variance s^2 indicating the closeness of fit of the auto-analyzer readings to the regression.

These experiments were performed at each of three concentrations of acid and at each of two reaction temperatures, in an attempt to establish the best conditions. The results are shown in Table 2 and illustrated in part in Fig. 9.

In order to discriminate adequately between raffinose and arabinose, the conditions giving low values for $\frac{b}{a}$ are advisable, and with an acid concentration of 40% at 98°C. the values were considered sufficiently low. However, for greatest sensitivity, the conditions giving the highest values of 'a' are advisable, and the results with the higher of these two acid concentrations were

TABLE 2

Interference of Arabinose with Raffinose Estimation

Technique Using Different Conditions

The optical density readings are expressed for convenience in units of 10,000 times optical density.

Conc. of HCl.	Temperature °C.	'a'	'b'	$\frac{b}{a}$ 100	S.e. of 'a' and 'b'	S^2
80%	98	427	300	70	11	1019
	90	317	92	29	5	220
60%	98	274	38	14	3	100
	90	132	8	6	3	104
40%	98	119	4	3	2	22
	90	34	3	9	1	20

Reagent contained 1% resorcinol in all cases

Sample flow rate: 2.0 ml./min.
Reagent flow rate: 4.5 ml./min.

considered the more favourable in this respect. A further consideration is the discrepancy between duplicate estimations for a standard solution, and Table 3 gives the results for the two raffinose standards estimated under the conditions already mentioned. The smallest discrepancies arise at the 60% acid concentration at a reaction temperature of 98°C., and these were the conditions employed in future experiments.

Resorcinol concentration effect

Solutions containing raffinose and arabinose, in the final concentrations shown below, were estimated in the presence of different concentrations of resorcinol contained in 60% hydrochloric acid reagent, at a reaction temperature of 98°C.

R_1 - 25 µg. raffinose/ml.; R_2 - 50 µg. raffinose/ml; R_3 - 75 µg. raffinose/ml.

R_1A_1 - 25 µg. raffinose and 25 µg. arabinose/ml.

R_2A_2 - 50 µg. raffinose and 50 µg. arabinose/ml.

These were run on the autoanalyzer in the order R_1 R_1 R_2 R_3 R_1A_1 R_2A_2, then the reverse order.

From the results it was possible to estimate (i) the increase in optical density caused by an increase in raffinose concentration of 25 µg./ml.

TABLE 3

Percentage Difference Between
Duplicate Readings for Raffinose
Standards Under Different Conditions

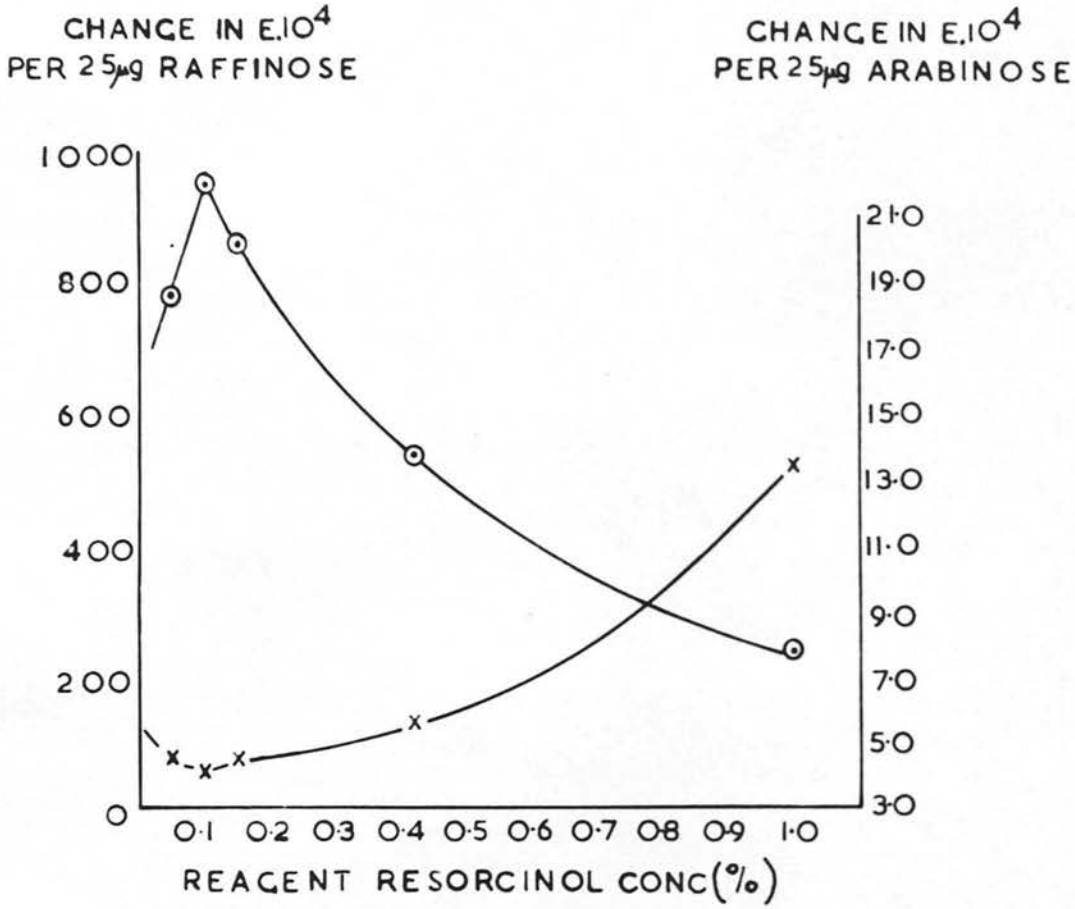
Temp.	Hydrochloric Acid Concentration					
	80%		60%		40%	
	R_1	R_2	R_1	R_2	R_1	R_2
98°C.	3.8	5.5	0.6	0.9	2.2	4.0
90°C.	5.0	1.0	0.6	3.2	4.9	0.0

R_1 25 µg. raffinose/ml.

R_2 50 µg. raffinose/ml.

FIG. 10.

EFFECT OF CHANGES IN RESORCINOL CONCENTRATION ON RAFFINOSE ESTIMATION TECHNIQUE



The points are plotted from the values in Table 4 for the average optical density changes measured on the autoanalyzer per 25 µg./ml. increase in raffinose, or arabinose concentration.

(ii) the change in optical density due to a 25 µg./ml. increase in the arabinose concentration. Under the most favourable conditions, (i) will be large and (ii) should be very small. The results are presented in Table 4 and Fig. 10. It is apparent that the resorcinol concentration of the reagent is a critical factor in the estimation of raffinose. The narrow peak at 0.1% resorcinol, where maximal raffinose colour is obtained, is associated with minimal interference from arabinose and this is the concentration adopted in future experiments.

Application of the technique to extracts of the rat heart

In his experiments estimating raffinose by the original Cole procedure, Zachariah (Ph.D. thesis Oxford) found raffinose-like substances present in the heart which caused erroneously high results for determinations of added, known amounts of the trisaccharide in heart extracts. An experiment was therefore performed to check if such compounds contribute to the raffinose colour as measured by the modified estimation procedure using the auto-analyzer. Three standard raffinose solutions

TABLE 4

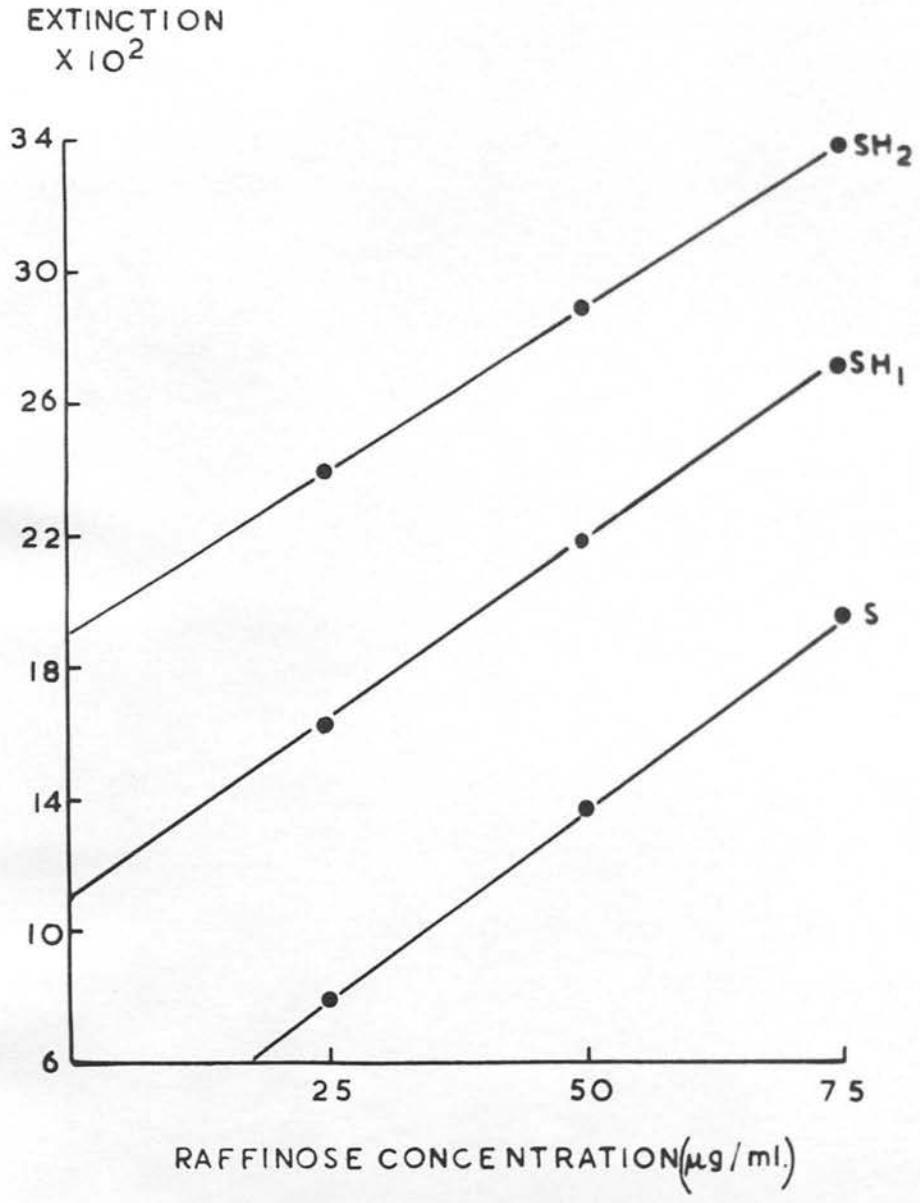
Effect of Changes in Reagent Resorcinol Concentration on Raffinose Estimation

	Resorcinol Concentration (g./100 ml.)				
	1.00	0.42	0.15	0.10	0.05
Raffinose slope	246.0	544.0	863.0	947.0	788.0
Arabinose slope	13.5	5.7	4.5	4.0	4.5

Sample flow rate: 2.0 ml./min.
Reagent flow rate: 4.1 ml./min.
Reagent Hydrochloric conc: 60%
Reaction temperature: 98°C.

FIG. 11.

ESTIMATION OF STANDARD RAFFINOSE SOLUTIONS IN THE PRESENCE OF HEART EXTRACTS



The points are the average extinctions of duplicate samples.

Line S is drawn through the raffinose standards
Line SH_1 is for raffinose standards with heart homogenate
Line SH_2 is for the standards with twice the concentration of heart homogenate given in SH_1.

were run on the autoanalyzer, first alone, and then in the presence of two different concentrations of heart extracts. These had been prepared by homogenising hearts which had been washed free of blood prior to addition of 2.6% cadmium sulphate and 4% sodium hydroxide to yield protein-free filtrates in the manner used after perfusion experiments. The heart-extract concentrations used covered the range likely to be encountered in perfusion experiments. Fig. 11 shows the results. Endogenous raffinose-like materials are clearly present, and a control experiment using water in place of heart homogenate confirmed that the effect is due to a true heart raffinose blank and not to turbidity resulting from the protein-precipitation procedure.

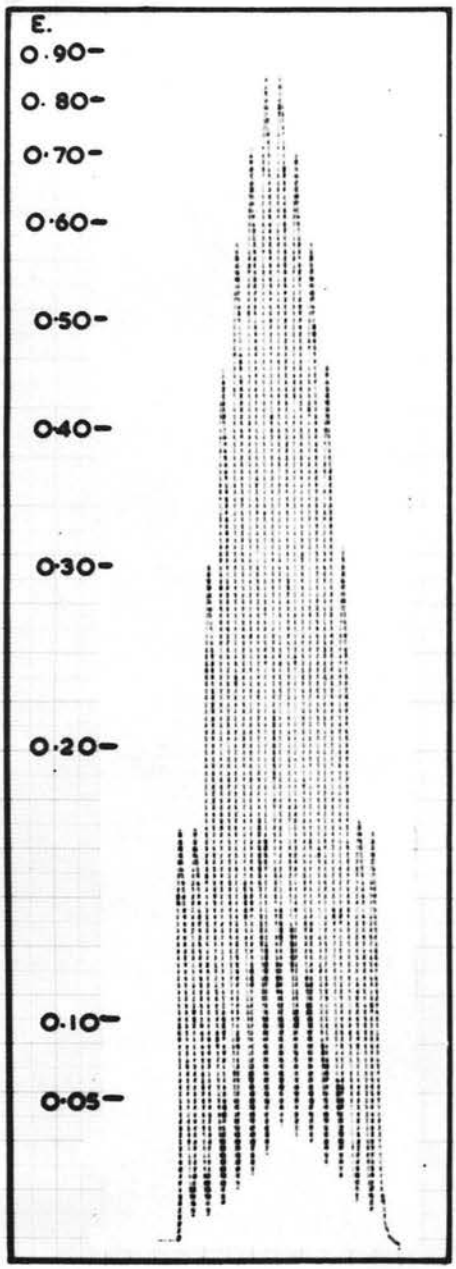
Similar experiments indicated that the tissue blank was of the order of 200-300 µg. per heart, and this did not alter whether hearts were immediately homogenised and analysed, or allowed to stand for up to 1 hr. at room temperature prior to homogenising. The raffinose to be estimated in each heart when it is used as an extracellular space marker is likely to be 1500 µg. so that errors of 20% might result if no allowance were made for the blank.

Attention was directed at getting rid of the substances contributing to the blank by using yeast. It was hoped that the simple endogenous heart sugars would be destroyed, permitting accurate determination of the extracellular marker. A suspension of washed yeast cells was prepared, and various mixtures of this with raffinose solutions and heart extracts incubated at 30°C. The experiments were unsuccessful - the treatment did not reduce the heart blank and the yeast itself contributed interfering chromogens. It became apparent, therefore, that severe disadvantages attended the use of raffinose to assess the interstitial volume of the perfused rat heart.

Another fructosan, inulin, has been widely used as an extracellular marker (Boyle, Conway, Kane and O'Reilly, 1941; Wilde, 1945; Bleehen and Fisher, 1956). It contains fructofuranose residues linked by β (2 1) glycosidic bonds, and in view of this structure, the technique devised for raffinose should prove adequate for inulin determination. In addition, a particular concentration of inulin should yield a more intense colour on treatment with resorcinol hydrochloric acid reagent than an equal concentration of raffinose since the latter contains

FIG. 12.

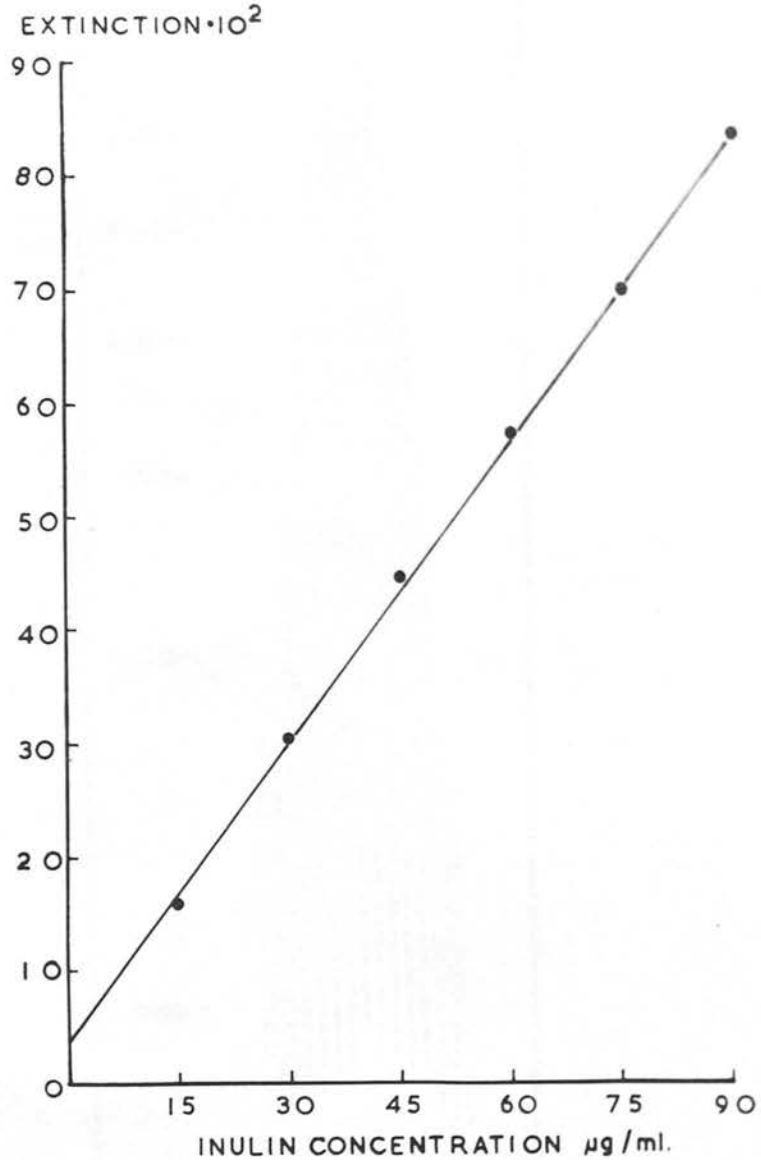
Inulin Estimation



Six standard inulin solutions (15,30,45,60,75 and 90 µg./ml.) were run on the autoanalyzer using the fructofuranoside technique and the reagent mentioned on p. 65.

FIG. 13.

INULIN ESTIMATION
AGREEMENT WITH BEER'S LAW



The points are the average extinctions from the duplicate peaks shown in Fig. 12. A regression line has been drawn through the points.

Only a slight adjustment of the recorder is required to permit this line to pass through the origin.

glucose and galactose residues as well as the fructose residue. Experiments were performed to test these possibilities.

Determination of Inulin

It was first essential to test the conformity of the inulin estimation to Beer's law. Six standard solutions, containing inulin in the concentrations shown below, were put through the fructofuranoside technique described earlier (p.52 The tracing obtained is shown in Fig. 12. The plot of the results in Fig. 13 shows the excellent agreement with the law over the range of concentrations studied.

Reagent:- concentrated hydrochloric acid containing 0.083 g. ferric chloride per litre, diluted to 60% strength with water and containing a final resorcinol concentration of 0.1%.

Samples:- Solutions containing 15, 30, 45, 60, 75 and 90 µg. inulin/ml.

Using the same estimation procedure, the following solutions were analysed:-

I_1 10 µg. Inulin/ml. I_2 20 µg. inulin/ml.

I_3 30 µg. inulin/ml. $K_4^{I_4}$ 40 µg. inulin/ml.

X 40 µg. inulin/ml. deproteinised by the cadmium hydroxide procedure

Y a deproteinised heart extract previously found to contain 7.22 µg. apparent raffinose material/ml.

R_1 25 µg. raffinose/ml. R_3 75µg. raffinose/ml.

The results are given in Table 5. They indicate that inulin gives an approximately three fold increase in optical density when compared with an equal concentration of raffinose, and the heart blank is consequently one third as great. However, the conclusion of Young (1960) that cadmium hydroxide precipitation removes inulin from solution is confirmed here.

A series of experiments was performed testing various protein-precipitants on heart homogenates and inulin-containing solutions. Addition of cadmium sulphate and equivalent quantities of sodium hydroxide always gave low heart blanks but always removed some inulin from solution. Neither perchloric acid nor trichloracetic acid reduced the apparent inulin content of the solutions, but both gave high heart blanks. Metaphosphoric acid was tested at various concentrations and it was

TABLE 5

Estimation of Inulin on the Autoanalyzer

Solution	I_1	I_2	I_3	I_4	X	Y	R_1	R_3
Inulin content µg./ml.	10	20	30	40	40	0	0	0
Apparent Inulin content µg./ml.	9.87	20.28	29.86	39.45	0.54	2.07	7.95	24.64

The inulin content of the solutions was estimated using the raffinose technique described on page 52. The meaning of the symbols is given on p. 66.

finally decided that this reagent, in a final concentration of 2%, gave the best results. Table 6 shows the results of an experiment testing the properties of this protein-precipitant in our system. It precipitated no inulin and left a heart blank of a tolerable level, 29 µg. in the heart of this experiment (see below for mean blank value). It was decided at this stage that the best course open was to estimate the apparent inulin contents of several rat hearts, perfused for different periods of time, and to correct for these amounts in future experiments.

Hearts were dissected out from male, albino rats, cannulated, washed free of blood, and perfused for various times with Krebs Bicarbonate Ringer containing 4mM sodium pyruvate to act as nutrient. At the end of the perfusions the hearts were blotted lightly on filter paper to remove excess perfusate and homogenised. These operations are very similar to those described later for the main perfusion experiments of this work. The homogenates were transferred to 25 ml. volumetric flasks, a known amount of inulin-containing solution added, the mixture deproteinised using metaphosphate in final concentration of 2%

TABLE 6

The Use of Metaphosphoric Acid as Protein Precipitant

Solutions Added				1 ml. 20% HPO_3	1 ml. 20% HPO_3 2 ml. Heart Homogenate
Inulin added (µg./ml.)	10.00	20.00	30.00	25.00	25.00
Inulin estimated (µg./ml.)	9.98	20.05	29.98	25.10	29.70

The heart homogenate contained 2 rat hearts in 25 ml. so that the heart inulin blank here is 29 µg. per heart.

and made up to the mark. After mixing thoroughly and centrifuging, the inulin contents of the supernatants were estimated on the autoanalyzer. The heart blanks, calculated from the difference between inulin added and inulin estimated in the solutions, are presented in Table 7. The values were extremely variable ranging from 6 µg. to 160 µg. Neither the size of the hearts nor the perfusion period consistently affected the blank value. The mean value is 71.5 µg. with a standard deviation of 32.0 µg. and this agrees well with the result of Young (1960).

When inulin is used as anextracellular space marker in the perfused heart, the total heart content is expected to be approximately 4500 µg. so that by ignoring the blank value, an error of 1 - 2% would result. However, subtraction of 71.5 µ g. from all estimated values of total heart inulin would incur a very small error, and it was felt that this procedure would be perfectly adequate for future experiments.

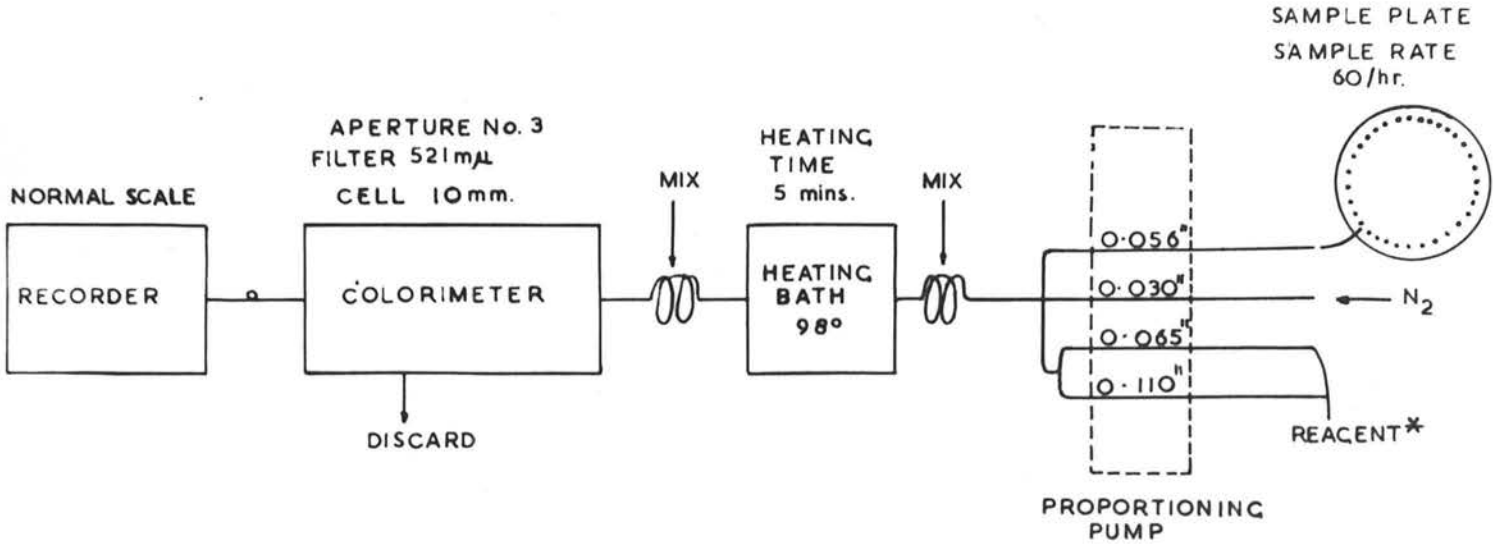
TABLE 7

Endogenous Inulin-like
Materials in Perfused Hearts

	Total Perfusion Time (min.)				
	0	30	40	90	120
µg. Inulin per heart	74.5 (9)	79.7 (1)	48.0 (5)	78.3 (9)	81.7 (2)

The number of observations is given in parenthesis

FIG. 14.



AUTOANALYZER SYSTEM—PENTOSE ESTIMATION

*Reagent:- 75% acetic acid containing 5% p-bromoaniline.

2. Pentose Estimation Using the Autoanalyzer

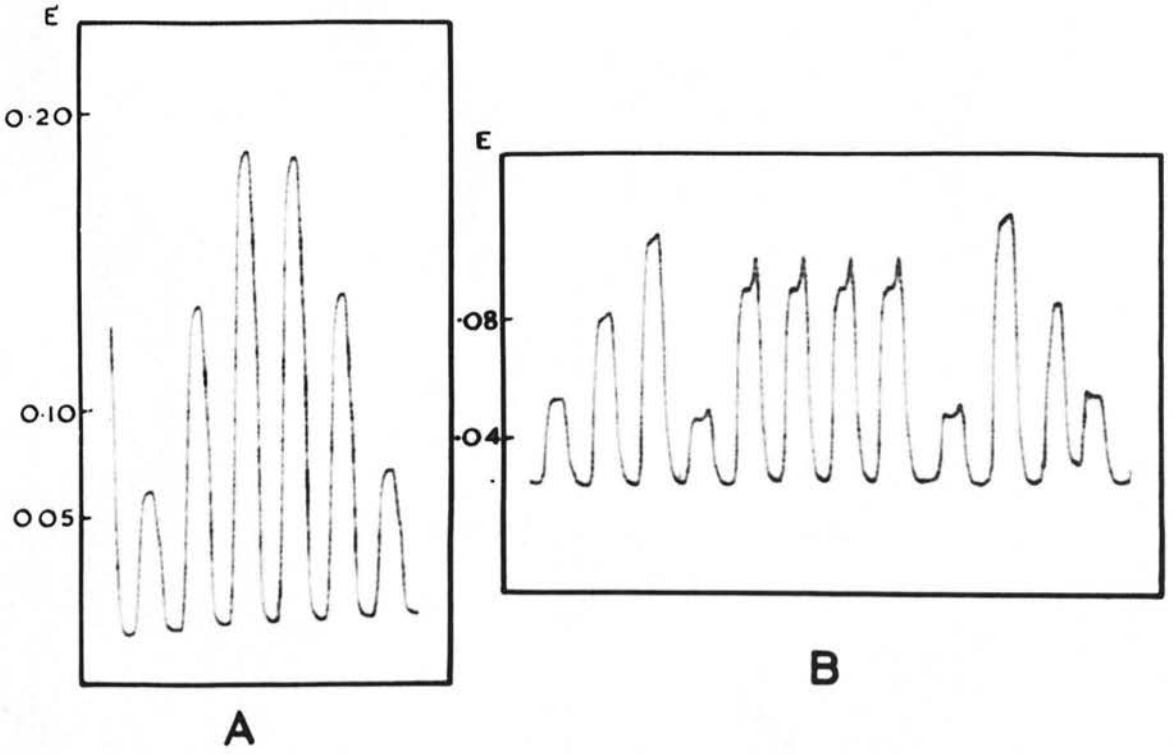
The basis of the method finally adopted was developed by Roe and Rice (1948). At 70°C. glacial acetic acid converts pentose sugars to furfural, and this yields a cherry-red colour with p-bromoaniline, the complex having an absorption maximum at 520 mμ. The glacial acetic acid should be saturated with thiourea which serves as an antioxidant, preventing the production of quinoid materials from the p-bromoaniline. Zachariah (1961) improved the sensitivity of the method by increasing the reaction temperature to 80°C. The heating time is 10 min. but mixtures must be kept in the dark for 100 min. after heating for maximum colour to develop.

Adaptation to the autoanalyzer and final procedure adopted

Before describing the modifications necessary in adapting the method to the autoanalyzer, the final procedure adopted will be described. Fig.14 shows the arrangement of the equipment in diagrammatic form. Aliquots (1.5 ml.) of the samples to be estimated and of the standard solutions are distributed on the sampler plate, together with

FIG. 15.

Pentose Estimations



The compositions of the solutions and the proportions of reagent to sample are given on P. 74.

Reagent A:- 75% acetic acid containing 4 g. p-bromoaniline/100 ml.
B:- Reagent A saturated with thiourea.

water cups, as described earlier (p. 50). The reagent is gassed with nitrogen for 1 hr. prior to the start of the analysis, when the tube to feed reagent into the system is placed in the reagent bottle. The nitrogen stream is now directed to just above the surface of the reagent, and the tube to feed nitrogen directly into the auto-analyzer system inserted into the gas space above the reagent. With the sampler tube dipping in water, the proportioning pump is started, and after a few minutes have elapsed, the sampler tubing is passed through the crook on the sampler plate, down to the bottom of the first cup, and the analysis begun by switching on the sampling device.

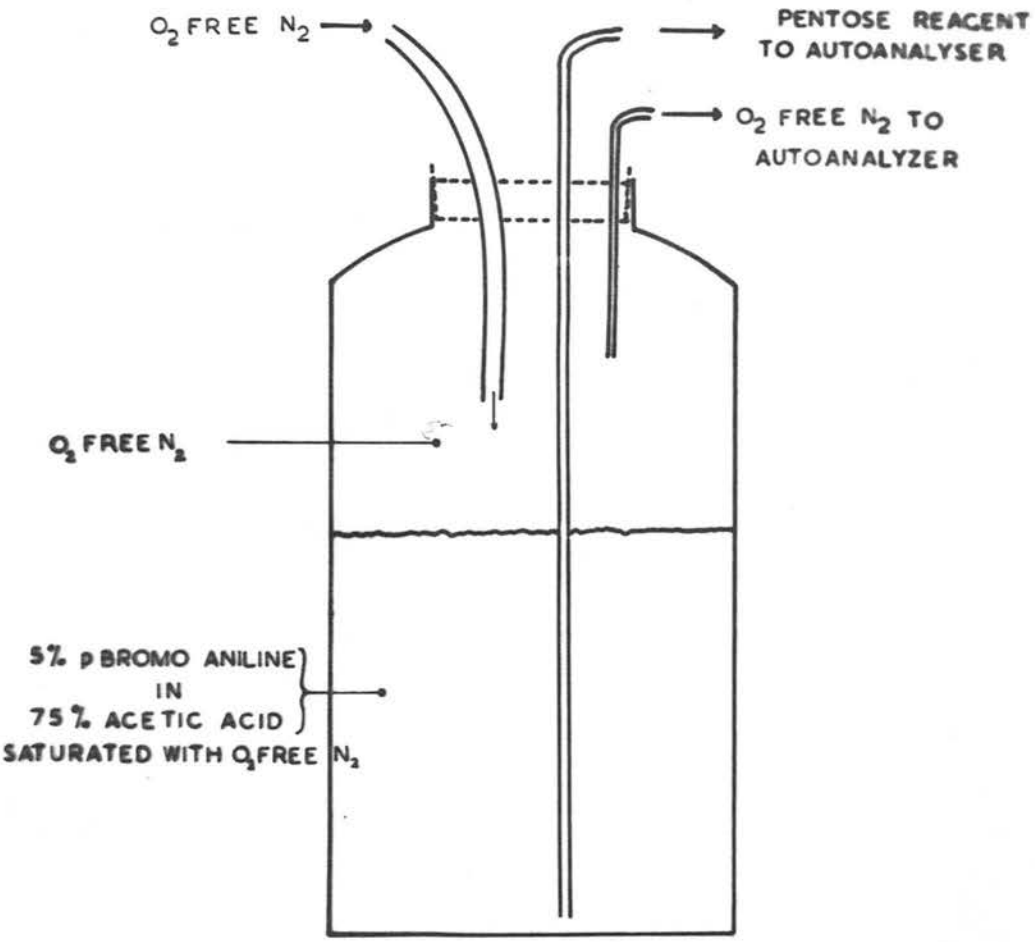
Modifications of the manual technique

As had been anticipated, the glacial acetic acid attacked the tygon tubing used on the auto-analyzer. Dilution of the acid to 75% strength permitted long life of the tubes without diminishing the sensitivity of the technique appreciably.

An interesting finding was that the high concentration of thiourea in the reagent severely depressed the colour intensity of the reaction mixture. Fig. 15 shows the results of an experiment testing the effect of thiourea in the reagent.

FIG. 16.

NITROGEN GASSING DEVICE



A - lacking thiourea
B - saturated with thiourea (6.5 g.%)

Sample speed 20 samples/hr.

Reagent flow rate 5.9 ml./min.

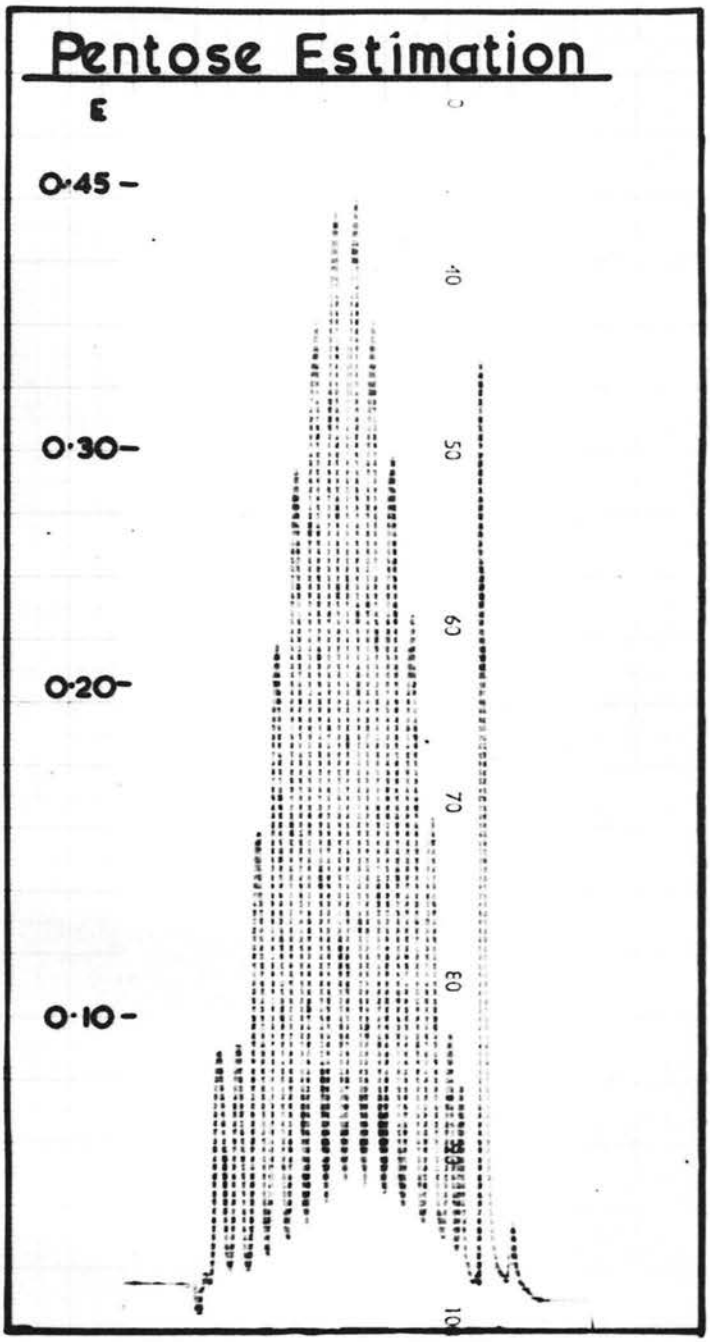
Sample flow rate 1.2 ml./min.

Air segmentation 0.32 ml./min.

Samples : solutions containing 15, 30 and 45 µg. arabinose/ml. and some 'unknowns' are included in trace B.

It will be seen that it is essential to include the antioxidant in the reagent if the upward drift of the trace is to be prevented. Accordingly, different concentrations of other antioxidants - hydroquinone and pyrogallol, were tried, but without success. It was then thought that instead of using an antioxidant, it might be possible to eliminate oxygen from the system. It was therefore decided to pass nitrogen gas into the system. This could replace the air bubbles previously used to isolate samples one from another and at the same time keep the reagent oxygen free. The apparatus employed for this purpose is shown in Fig. 16. The arrangement was completely successful and permitted the combination of the stable base-line associated with lack of reagent oxidation with the higher peaks resulting from the

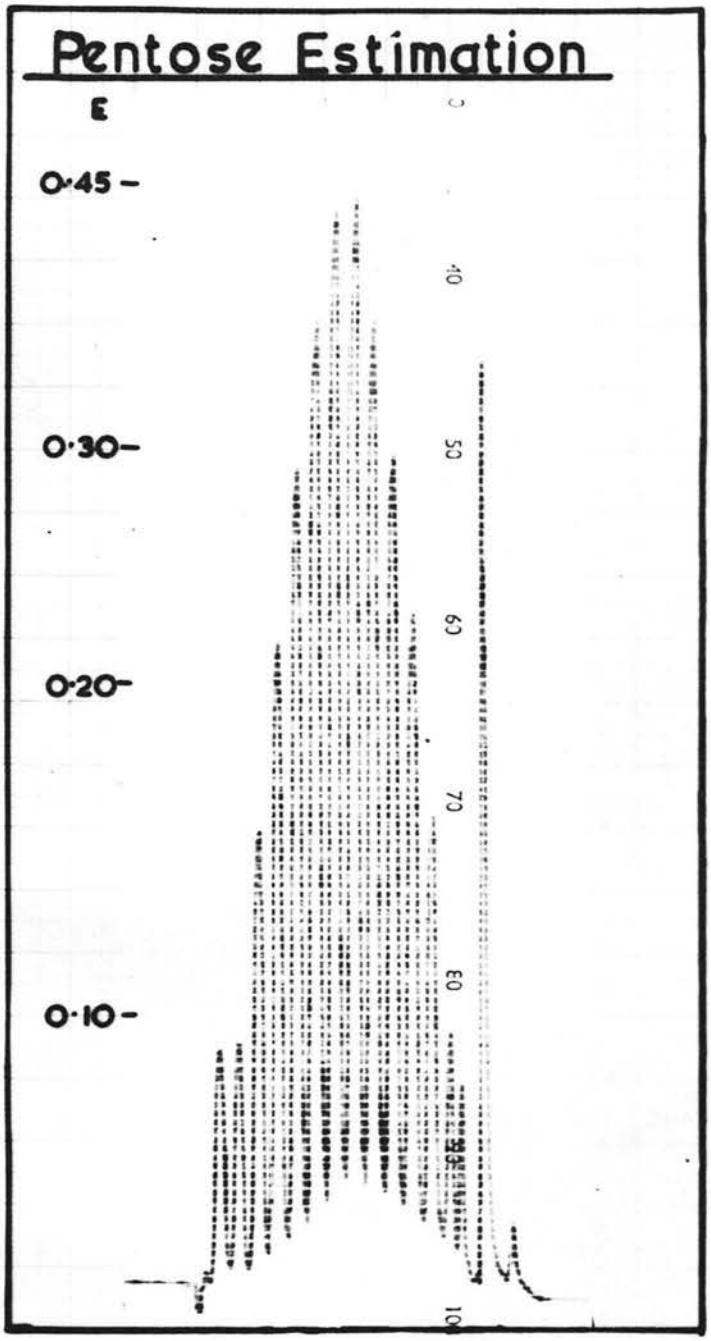
FIG. 17.



Solutions containing 50, 30, 45, 60, 75 and 90 µg. Xylose/ml. were analysed by the pentose estimation procedure described on p.

Reagent - 75% acetic acid containing 5 g. p-bromoaniline/100 ml.

FIG. 17.

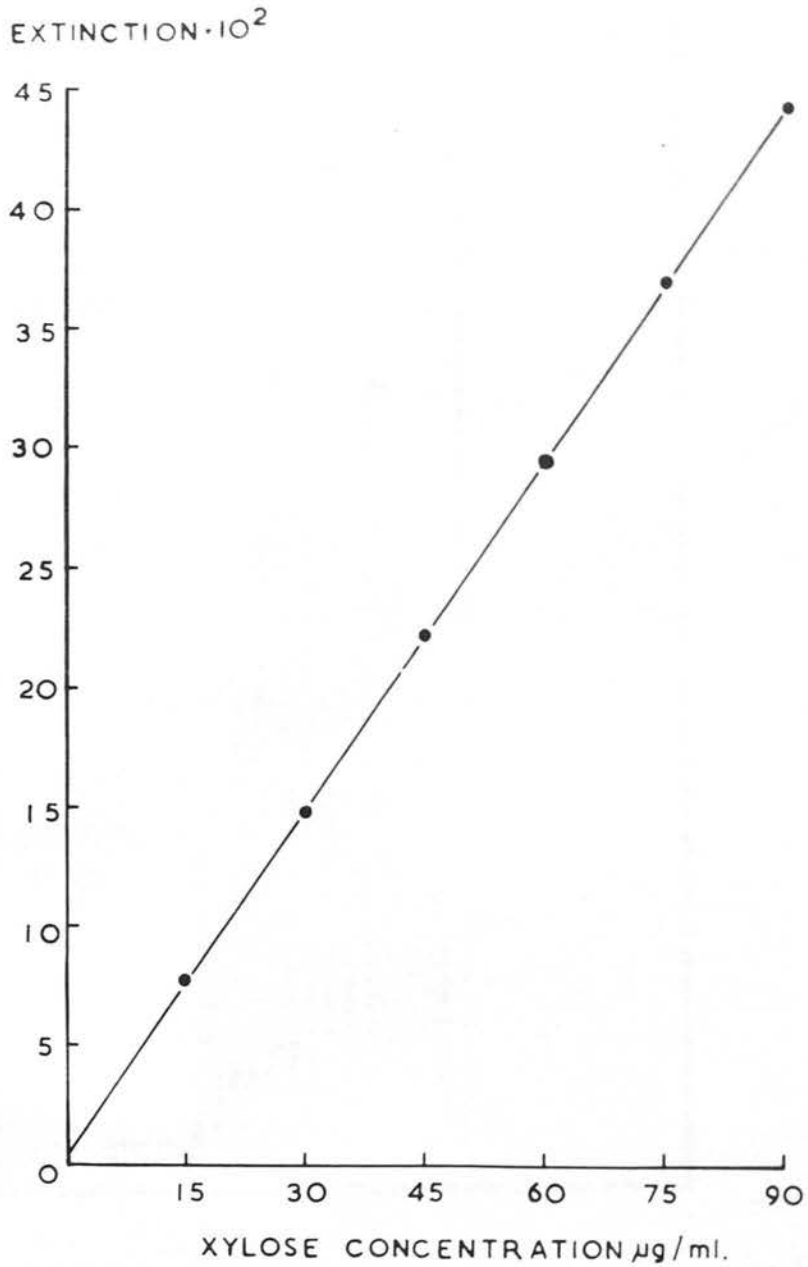


Solutions containing 50, 30, 45, 60, 75 and 90 µg. Xylose/ml. were analysed by the pentose estimation procedure described on p.

Reagent - 75% acetic acid containing 5 g. p-bromoaniline/100 ml.

FIG. 18.

PENTOSE ESTIMATION
AGREEMENT WITH BEER'S LAW.



The points are the average extinctions of duplicate samples shown in Fig. 17. A slight adjustment of the recorder is required to make the regression line pass through the origin.

exclusion of thiourea. The peak heights were further increased by increasing the p-bromoaniline concentration of the reagent to 5%. The reagent developed finally is:- 75% acetic acid containing 5% p-bromoaniline.

Using this reagent, and the autoanalyzer arrangement given earlier (p. 72) standard pentose solutions containing 15, 30,45, 60, 75 and 90 µg. xylose/ml. were put through the procedure in order to test conformity to Beer's law. Fig. 17 shows the tracing obtained, and a plot of the results is given in Fig. 18. There is excellent agreement with Beer's law over the range of concentrations studied.

Sources of error

It was found that the presence of inulin in a solution in no way interfered with the estimation of the pentose.

A difficulty was encountered, however, when the method was used on protein-free heart extracts. The metaphosphoric acid technique described earlier precipitated p-bromoaniline from the system making pentose determination impossible. Cadmium hydroxide had no such effect, and proved a satisfactory protein precipitant. This finding

necessitates that any heart homogenate be divided into two portions for inulin and pentose determinations, one portion being deproteinised with metaphosphate (for inulin), the other with cadmium hydroxide.

The possibility that endogenous heart materials might interfere with the pentose estimation was investigated as follows - 4 hearts were perfused with a sugar free medium for 60 min. prior to homogenisation. A known quantity of pentose was added to each homogenate, and after precipitating the protein the pentose contents of the solutions were estimated. The pentose equivalents of the hearts gave a mean value of 6.3 µg. per heart, and since preliminary perfusion experiments with short perfusion times, indicated that the minimal pentose uptake by the heart was about 500 µg., the error incurred by ignoring such a blank will be approximately one per cent.

CHAPTER IV

Comparison of Manual and Automatic Procedures

A check on the relative efficiences of the manual and automatic techniques was made as follows. For any set of estimations three standard solutions have been used, and the optical density given by these with any method has always been determined in duplicate. To obtain the sugar content of an unknown solution, a regression line has been computed from the values given by the standards. For any analysis, it is possible to compute the mean values for all three standards using the best fit regression line. The deviations of these means from the theoretical amounts of sugar in the standard solutions are given in Table 8 ($x - \overline{x}$ µg.), for arabinose and raffinose determinations by both manual and automatic techniques. The actual amounts of sugar in the standard solutions are given at the top of the table and at the foot are given the standard deviations and the coefficients of variation. Both of these are smaller for the automatic than for the manual technique.

TABLE 8

Comparison of Manual and Automatic Estimation Procedures

	Arabinose		Raffinose	
	Automatic	Manual	Automatic	Manual
Standard Solutions used (µg./ml.)	15.01 30.03 45.04	18.02 36.04 54.06	25.00 50.00 75.00	23.78 47.56 71.34
	$(x-\bar{x})$ µg.	$(x-\bar{x})$ µg.	$(x-\bar{x})$ µg.	$(x-\bar{x})$ µg.
	0.12	0.25	0.19	0.30
	0.26	0.52	0.38	0.75
	0.09	0.27	0.19	0.30
	0.01	0.04	0.08	0.05
	0.04	0.06	0.12	0.17
	0.05	0.02	0.08	0.01
	0.08	0.27	0.03	0.32
	0.12	0.21	0.02	0.45
	0.09	0.21	0.03	0.26
	0.01	0.22	0.11	0.22
	0.00	0.30	0.23	0.44
	0.00	0.16	0.11	0.22
	0.10	0.02	0.22	0.11
	0.19	0.05	0.43	0.23
	0.10	0.03	0.22	0.11
Coefficient of variation	0.63%	1.06%	0.70%	1.16%
Standard deviation (µg.)	0.19	0.38	0.35	0.55

CONCLUSION

In this section a machine has been described which was designed for the routine estimation of comparatively large amounts of compounds. It is used, for instance, for determinations of blood cholesterol or glucose levels, where the amounts to be estimated are likely to be 2 mg./ml. and 1 mg./ml. respectively. In the present work its application has been extended to the estimation of pentose sugars and fructofuranosides where the final concentration is some five hundred times smaller than those mentioned above.

A significant feature of the autoanalyzer is the identical treatment of all the samples introduced into the system throughout each procedure. As a result, even colours of low intensity resulting from very short heating times for reaction mixtures can be determined with adequate precision. This means that techniques which at first appear unsuitable for automation, as, for instance, does the Roe and Rice pentose method involving the standing in the dark of reaction mixtures for 100 min., can easily be modified and applied in this system.

Advantages worth consideration include the greater speed of the automatic technique and the freedom of the experimeter to do other things once an estimation has been set in motion. In view of the number of determinations involved in the present research programme, operator fatigue is a very real factor which is avoided by automation, and in addition, the less elaborate preparation for any established method and the ease of cleaning out of analytical apparatus provide added advantages.

The use of the machine has, of course, been accompanied by disadvantages and in particular the necessity of diluting the more corrosive reagents, and the much shorter heating times should be mentioned. With the estimations involved here, these drawbacks have been overcome, and the sensitivity of the methods has been enhanced rather than diminished by the modifications in both techniques. Thus the comparison of the manually performed methods with the automatic ones reveals the greater precision of the latter for both pentose and fructufuranoside estimations. It was therefore felt that the use of the autoanalyzer throughout the present study was justified.

CHAPTER V

Summary

1. A machine has been described which is considered suitable for the automatic estimation of micro amounts of pentose sugars and fructofuranosides.

2. In employing this machine - the Autoanalyzer, extensive modifications have been necessary of pre-existing manual methods used for the estimation of the compounds mentioned.

3. Considerable difficulties were encountered when attempts were made to estimate raffinose in protein-free extracts of the rat heart, and it was found that it would be advantageous to use inulin instead of raffinose to measure the extracellular space of the perfused heart.

4. A comparison of the value of the manual and automatic estimation procedures for the pentoses and the fructofuranosides indicated that the automatic procedures have several advantages, and these are associated not only with the ease of setting up and performing the techniques but also with their increased sensitivity.

SECTION III

EXTRACELLULAR SPACE MEASUREMENT AND PERMEABILITY CHANGES IN THE PERFUSED HEART

CHAPTER I

Introduction

The measurements of sugar uptake to be made in the experiments of the later sections of this thesis depend upon an exact estimation of the amount of sugar transported into the cells of the perfused rat heart after different times of perfusion. The amount of sugar taken up by the organ has therefore to be precisely partitioned into that present in the cells and that filling the interstitial fluid. The intracellular volume of a tissue is usually determined indirectly, by first estimating the volume of the interstitial fluid and subtracting this from the total tissue water. The first problem, therefore, is to establish a convenient and reliable technique for the determination of the interstitial fluid of the perfused heart.

Tissue 'spaces'

In what follows the term 'space' will be used to mean the ratio of the amount of some solute found in a tissue, or in a compartment of a tissue, to the amount of the same solute in unit volume of the perfusate flowing through the blood vessels of the tissue.

The 'space' so determined has the dimensions of a volume. If the volume of fluid in the tissue accessible to the solute is freely accessible, then the concentration of solute in it will rapidly become equal to that in the perfusate: in these circumstances the 'space' will be equal to the volume of fluid which is accessible to the solute.

Where permeation is slow, and where equilibration of the water of the compartment with the solute is not complete at the time of the determination of the 'space' it is possible to use the measurement of 'space' to determine the mean concentration of solute in the compartment.

In this work, we need to know mean concentrations of slowly penetrating sugars in the cardiac muscle cells. If the 'space' of a penetrating sugar is known and if we also know (a) the total

water of the tissue and (b) the 'space' of some substance which does not penetrate the cells (which will be called the 'extracellular marker') then we can determine the mean intracellular concentration of the penetrating sugar.

The steps are:

(1) The space of the extracellular marker is determined.

(2) The difference between the space of the total tissue water and the space of the extracellular marker equals the intracellular water content.

(3) The difference between the space of the penetrating sugar and that of the extracellular marker represents the volume of perfusate which would contain the amount of penetrating sugar which has entered the cells.

(4) The ratio of this volume to the volume of intracellular water is equal to the ratio of the intracellular concentration of penetrating sugar to its concentration in the perfusate. This ratio is referred to as 'f' the fractional penetration of cell water by the sugar. In tables given later, the ratio is also given as the percentage penetration of cell water, where all values of 'f' are multiplied by 100.

At the outset of this work it was intended to use raffinose as the extracellular space marker. It has been used in ascites tumour cells, (Crane, Field and Cori, 1957) and in the isolated rat diaphragm, (Helmreich and Cori, 1957) and it was used in the perfused rat heart by Fisher and Zachariah (1961) in the experiments providing the background to the present work. However, in Section II it was shown that difficulties were encountered when attempts were made to estimate raffinose in heart extracts by automatic analytical methods. These difficulties could be avoided if inulin, which was employed as an extracellular space marker by Bleehen and Fisher, (1954) was used instead of raffinose. In this section, experiments are described to test the value of inulin as an extracellular space marker. In this connection some fundamental requirements of such a marker should be mentioned.

(i) The marker should not penetrate the cells of the preparation.

(ii) It must equilibrate rapidly with the interstitial water.

(iii) Its volume of distribution should not be affected by the presence of other compounds in the perfusion medium.

These requirements receive consideration here, and in addition, the inulin space is compared with other possible measures of the extracellular space. The first of these is the raffinose space method which has been mentioned. The second is an empirical method, entirely different in principle from the chemical methods, which has been shown by Fisher and Young (1961) to give results in agreement with those given by the raffinose and sorbitol spaces.

Electrolyte distribution in the heart

In the course of the experiments to be described, it became apparent that it would be profitable to determine the distributions of certain electrolytes in the heart. Zachariah (1960) found that in hearts perfused with modified Krebs bicarbonate medium, the chloride ion was not only distributed throughout the interstitial fluid, but also penetrated the muscle cells. This is not the case in freshly excised muscle where the chloride ion is confined to the extracellular fluid

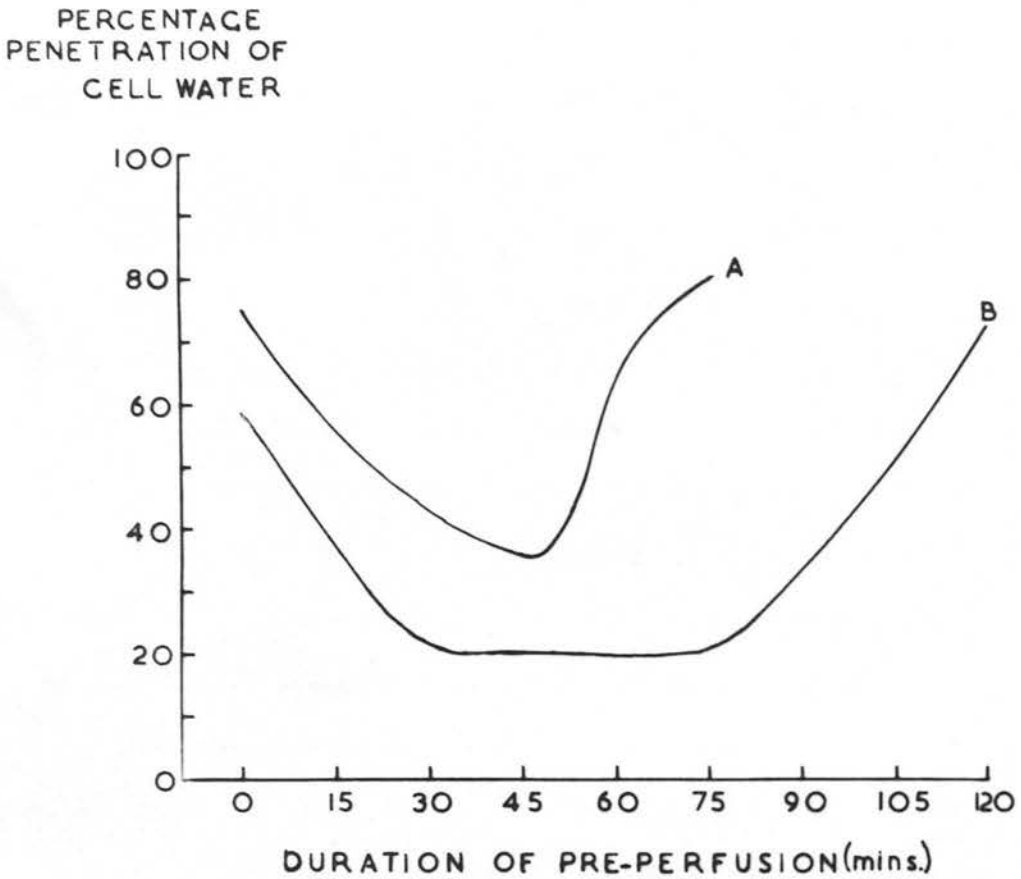
(Fenn, Cobb and Marsh, 1934; Eggleton, Eggleton and Hamilton, 1937). There can be little doubt that compared to its condition *in vivo*, the state of the heart when it is perfused is grossly abnormal. Nevertheless, the abnormal distribution of the chloride ion in the perfused heart was found by Zachariah to be reasonably constant, suggesting that the permeability of the cells of the preparation to this ion was stable.

Where the potassium ion is concerned, Zachariah (1960) obtained an intracellular concentration of approximately 150 mEq./l at the start of a perfusion, and this value is in good agreement with that found by Creese (1954) for the cells of the isolated rat diaphragm. However, as the period of perfusion of the heart was increased, Zachariah found that the intracellular potassium concentration fell and potassium ions were lost from the heart cells. There was therefore, a loss of potassium from the cells as the failure of the heart became imminent.

In the present investigation, therefore, it will be of interest to determine the distributions of potassium and chloride in the perfused heart, for these should give some indication of the state of the preparation.

FIG. 19.

CHANGES IN PERMEABILITY TO L-ARABINOSE



Hearts were preperfused for the periods shown and then perfused for 15 min. with 30mM L-Arabinose.

A - Krebs bicarbonate medium with half the recommended calcium and magnesium concentrations

B - The same medium with 4 ml. dialysed ox serum per 100 ml.

The curves are taken from the paper of Zachariah, 1961.

Permeability changes in the perfused heart

It was clearly demonstrated by Zachariah (1961) that the permeability properties of the perfused heart change during the life of the preparation. Thus, if hearts were perfused for different periods of time on a modified Krebs bicarbonate medium devoid of pentose, and then placed on a pentose-containing medium for 15 min. different results were obtained for the penetration of the cells by the sugar (Fig. 19 curve A). The fall in cell permeability over the first 30 min. of perfusion is thought to be due to the loss of endogenous insulin activity from the heart, since the permeability of fresh hearts from rats treated with anti-insulin serum, which depletes circulating insulin without damaging the β-cells of the islets, (Wright, 1959) is approximately the same as the permeability at the 30 min. time, (Zachariah, 1961). Such perfused hearts show no diminished sensitivity to insulin, and addition of the hormone to the perfusate elicits a reproducible response in sugar-uptake by the heart cell. The fall in sugar permeability is followed almost at once by an increase, and this is associated with a gradual diminution in the

force of contraction of the heart, complete failure occurring after 80 or 90 min. Zachariah (1961) was able to delay the changes associated with heart failure, and to obtain a 60 min. period of stable permeability (Fig. 19 curve B) by including dialysed ox-serum in the medium perfusing the heart. Such a period of stable permeability to sugars is essential for valid quantitative studies on sugar transport and so, in spite of the disadvantage of including ox-serum of unknown composition in the perfusate, this was the method used in the early experiments of the present work. However, it was found that the ox-serum could be omitted from the perfusate, and the hearts still maintained for several hours of perfusion if the sintered glass filter used by Fisher and Zachariah (1961) was replaced by a Whatman No. 50 paper filter. This modification in the perfusion technique, which involved a significant change in the composition of the medium perfusing the hearts, demanded that the permeability properties of the hearts be re-investigated under the new conditions. This investigation is included in this section

CHAPTER II

Materials and Methods

Animals

Male, albino rats weighing between 170 g. and 220 g. were used. In view of the finding by Young that there is a seasonal variation in the response of hearts to insulin, these animals were kept in conditions of constant temperature and day length (12 hr.) for a minimum of two weeks prior to use. Free access was permitted to food and water.

Perfusion medium

This was similar to the bicarbonate medium of Krebs and Henseleit (1932) but the final concentrations of calcium and magnesium were halved to allow for the binding of these ions by plasma proteins (Greene and Power, 1931). Fresh perfusate was prepared when required from the stock solutions kept in the cold room. It was found convenient to add the extracellular marker compound to a large volume of the perfusate and then to add the sodium pyruvate (to act as nutrient - Zachariah, 1960) before dividing the solution into

two portions and adding the required amount of test-sugar to one of these. The composition of the medium is shown in Table 9. The pH of the medium is 7.4.

Reagents

All the reagents used were analar quality, obtained from British Drug Houses Ltd. Doubly distilled water was always used for the media. When marked frothing of the perfusate occurred, it was reduced by touching the surface with a glass rod carrying a thin smear of Silicone Anti-foam A emulsion obtained from Hopkin and Williams Ltd.

Perfusion technique

The technique used for the perfusion of the isolated rat heart is based upon the closed circuit perfusion system of Locke and Rosenheim, (1907-1908). It incorporates the improvements made to that system by Bleehen and Fisher (1954) and is very similar to the technique used by Fisher and Zachariah (1961). The coronary circulation of the excised heart is perfused through a cannula in the aorta.

TABLE 9

The Composition of the Perfusate

Component	Concentration (mM)	Osmotic Coefficient	Active Milli. Osmolarity	Solids mg./ml.
NaCl	118.480	0.93	220.373	6.93
$NaHCO_3$	24.876	0.96	47.762	2.09
KCl	4.739	0.92	8.720	0.35
KH_2PO_4	1.186	0.87	2.064	0.16
$MgSO_4 7H_2O$	0.593	0.58	0.688	0.15
$CaCl_2 6H_2O$	1.270	0.86	3.277	0.28
		T O T A L	282.884	9.96

In addition to these basic solids, Inulin in concentration 2% contributes 3.33 m. osmols. to the osmotic activity and 20 mg./ml. to the total solids, bringing this latter value to 29.96 mg./ml. while 4mM sodium pyruvate is always added, bringing the final solids to 30.40 mg./ml.

FIG. 20.

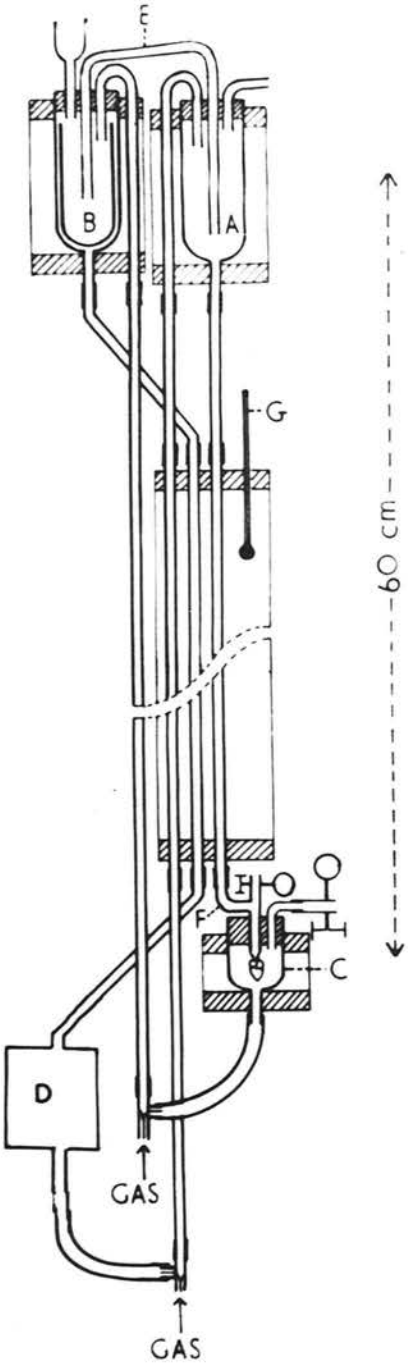


DIAGRAM OF

PERFUSION APPARATUS

A - Heart reservoir

B - Filter reservoir

C - Heart chamber

D - Filter

E - Siphon

F - Cannula and bubble trap

G - Thermometer.

Experiments for the study of permeability require two perfusion units of the type shown in Fig. 20. The cannula bearing the heart is placed in chamber 'C' so that the heart is completely immersed in perfusate. Perfusate from the reservoir 'A' passes down through the heart, the perfusion head being some 60 cms. of water. The effluent from the heart is lifted by a gas mixture containing 95% oxygen and 5% carbon dioxide to reservoir 'B'. This reservoir is situated above a filter at 'D' at a sufficient height (75 cms.) to ensure an adequate filtration pressure. A second gas lift carries the filtered medium back to reservoir 'A' for recirculation. The entire apparatus is jacketed so that the temperature of the perfusate and heart is maintained at 37°C. The volume of perfusate used was usually 60 - 70 ml. and this was always allowed to circulate at about 40 -50 ml./min. for 10 min. prior to the introduction of the heart, so that the liquid was adequately filtered and oxygenated. Before investigating permeability kinetics it was necessary to perfuse the heart for some time to establish a state of stable permeability (see Fig. 19). At the end of

the first perfusion, (preperfusion) the cannula bearing the heart was transferred to a second apparatus in which a different perfusate was circulating.

Filtration

Early in the present work, because of the inconvenience of cleaning and manipulating the sintered glass filters used by Fisher and Zachariah (1961) experiments were performed to test a filter made from Whatman No. 50 filter paper. Hearts were perfused with a Krebs bicarbonate medium containing pyruvate to act as nutrient, and it was found that the heart rate and force of contraction were maintained for several hours, whereas the hearts would have become markedly hypodynamic within 1 hr. if they had been perfused with this medium using a sintered glass filter in the circuit. Thus it was found that the dialysed ox-serum used in the perfusate by Fisher and Zachariah (1961) to maintain the hearts perfused in the apparatus including a sintered glass filter could be dispensed with if a paper filter was used. This finding will be discussed later.

FIG. 21.

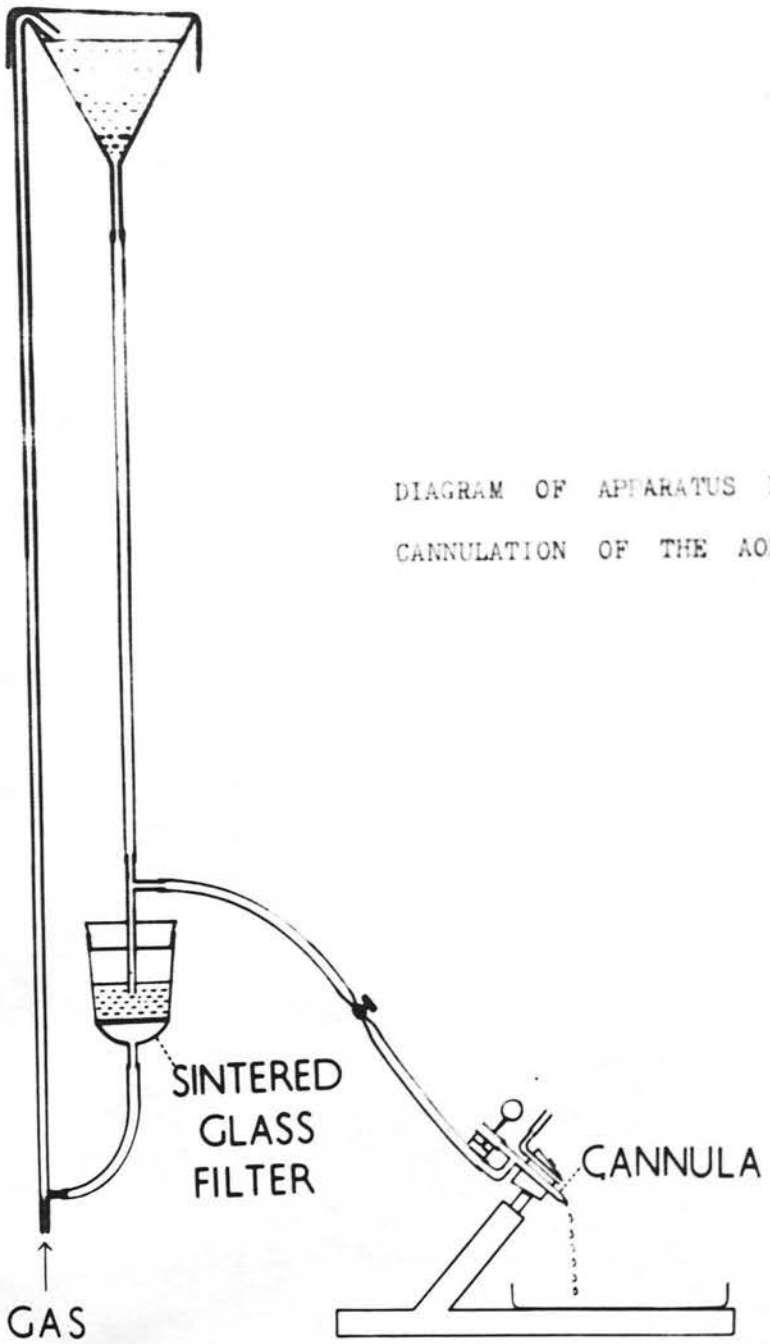


DIAGRAM OF APPARATUS FOR CANNULATION OF THE AORTA

Experimental procedure

The apparatus used during the cannulation of the heart is shown in Fig. 21. The circuit was filled with normal saline which was circulated at room temperature. Saline leaving the reservoir was filtered by the sintered glass filter and was then returned to the reservoir by a gas lift employing a mixture of 95% oxygen/5% carbon dioxide so that the saline was adequately oxygenated. A glass cannula was placed in the position shown and a slow drip of saline through it was started just before anaesthetising the animal. Ether was used to induce anaesthesia, and when this had been accomplished the thoracic cage of the rat was opened and the heart rapidly excised in such a way that a short length of aorta was still attached. The heart was placed immediately in a dish of saline and agitated to remove blood. The saline was cooled to reduce the chance of blood clotting and to reduce the metabolic activity of the heart during the brief period of anoxia. Any cleaning and freeing of the aorta was performed rapidly at this stage.

The aorta was next slipped over the tip of the cannula on the apparatus of Fig. 21. It was firmly tied in place with linen thread, and the screw clip controlling the flow of saline to the heart was fully opened. Within 1 min. all the blood of the heart had been washed out, and the cannula bearing the heart was then transferred to the main perfusion circuit (Fig. 20.).

Ligature tier

A useful adjunct to this procedure is a ligature-tier, for which I am indebted to Professor Fisher. This is similar in form to a pair of scissors, except that when the handles are brought together the 'blades' separate. Each 'blade' carries at its tip a slot into which the end of a ligature can be wedged. To use it, a ligature was tied in a knot with a large open loop big enough to slip over the heart, and the two free ends were wedged in the ends of the ligature tier blades. With the ligature-tier held in one hand, as one would hold a pair of scissors, it was easy to slip the loop of the ligature over the heart, which was held on to the aorta by a pair of forceps in the other hand. Bringing

together the fingers holding the tier then tied the ligature. The ends of the ligature can be cut close to the tier blades and a second knot can by tied by hand for security.

Preparation of heart homogenate

At the end of a perfusion, the ligature binding the heart to the cannula was severed and the heart was placed on a piece of hardened (Whatman No. 54) filter paper. The atria were removed by cutting along the atrioventricular septum and discarded, and both ventricles were divided from base to apex by a single cut. The two pieces of the ventricles were then blotted gently on the filter paper and placed in a tared bottle. After weighing the tissue, it was transferred to a small bottle and a little distilled water was added. The tissue was then minced using a pair of scissors and homogenised in an M.S.E. top drive homogeniser. The homogenate was transferred to a 25 ml. volumetric flask and made up to the mark.

Dry weight determination

Aliquots of the heart homogenates (10 ml.) were pipetted into stoppered glass weighing

bottles which were placed in an oven at a temperature of 105°C. for at least 24 hr. The bottles were then removed, cooled in a desiccator and weighed. The dry weights of the whole hearts were readily calculated from the weights of the dried homogenate samples.

The reproducibility of this method was checked by using it to determine the dry weights of 6 samples of a homogenate prepared from two large rat hearts. The final concentration of heart material in this homogenate was similar to that encountered in the main perfusion experiments. The coefficient of variation of the sample weights was 0.94%. No change in weight was found when samples were heated overnight for a second time and reweighed.

Procedure for the determination of pentose in the heart and perfusate heart sample

Heart Sample

A 5 ml. sample of the heart homogenate was placed in a 10 ml. volumetric flask and 1.5 ml. of 2.6% cadmium sulphate and two drops of phenol phthalein were added. Then 4% sodium hydroxide solution was added dropwise to the mixture until the phenol phthalein was permanently coloured.

After making the mixture up to volume, it was shaken thoroughly and centrifuged in an M.S.E. Minor Centrifuge. The supernatant was then analysed for pentose, (see p. 72).

Perfusate sample

A 1 ml. aliquot of the perfusate was placed in a 100 ml. volumetric flask and 20 ml. of distilled water added. The solution was now deproteinised using the procedure outlined above except that the quantity of cadmium sulphate employed was 2.5 ml.

Procedure for the determination of inulin in the heart and perfusate

Preliminary experiments indicated that if the cadmium hydroxide precipitation procedure was used for inulin-containing solutions, quite large quantities of inulin could be brought out of solution. It was therefore necessary to employ a different method of protein precipitation.

Heart sample

A 5 ml. sample of heart homogenate was placed in a 25 ml. volumetric flask and deproteinised by the addition of 2.5 ml. of freshly prepared 20%

solution of metaphosphoric acid. The solution was then made up to volume, mixed, centrifuged, and the inulin content of the supernatant was determined, (see below).

Perfusate sample

A 1 ml. aliquot of the perfusate was placed in a 500 ml. volumetric flask and approximately 20 ml. of distilled water added. The solution was deproteinised using the metaphosphate solution as outlined previously, and made up to volume. After centrifuging a sample, the supernatant was analysed for inulin.

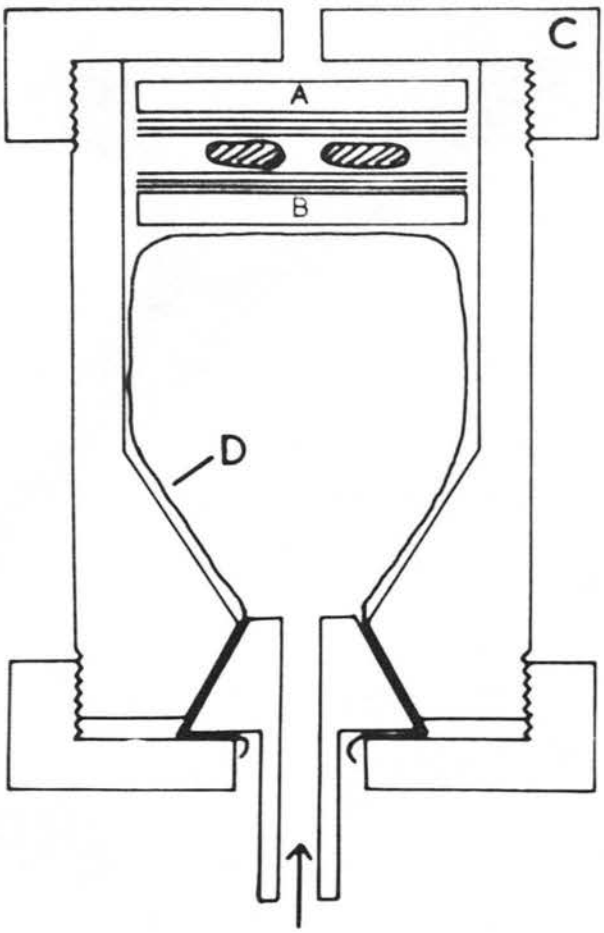
Procedure for determination of raffinose

The methods used in the preparation of the protein-free solutions were the same as those described for the determination of pentose.

Analysis of protein-free solutions

Samples (1.5 ml.) of the protein-free solutions obtained as described above were placed in autoanalyzer cups and the pentose, inulin or raffinose contents of the samples estimated as indicated in Section I.

FIG. 22.



The apparatus for determining the extracellular space by compression.

A. & B. Perspex discs between which the heart and filter paper circles are placed

C. Screw cap

D. Rubber balloon

For all estimations, the percentage transmission readings of the peaks recorded on the autoanalyzer were transposed into logarithms and then subtracted from 2.0000 to obtain the corresponding optical density readings. A regression analysis was performed on each set of results in order to calculate the pentose, inulin or raffinose content of the solutions.

Technique for determining the extracellular space of the heart by compression

The apparatus is shown in Fig. 22. It consists of a perspex cylinder which is closed by a screw cap at each end and it contains an inflatable balloon. The balloon is firmly fastened to an inlet tube passing through the lower screw cap of the cylinder. The balloon can be inflated by way of the inlet tube and it then presses a perspex disc against the upper screw cap. It will therefore compress any object between the disc and the cap. The balloon is connected through a three-way tap to the atmosphere on one side and to a source of compressed air on the other. A side tube on the line carrying the compressed air is connected to a mercury manometer

so that the pressure applied to the chamber contents can be measured. Nicks cut in the edge of the disc 'A' allow air to escape from the cylinder through the hole in the centre of the top screw cap. The two pieces of heart ventricular tissue obtained and weighed as described on page were placed between two 3 cm. filter paper circles (Whatman No. 54) and above and below these circles were placed three 3 cm. Whatman No. 1 filter papers. The papers and heart fragments were placed between the two perspex discs and set on the partially inflated balloon in the perspex cylinder with the bottom screw cap in position. The top screw cap was firmly screwed down and a pressure of 500 m.m. of mercury applied for 15 min. by inflating the balloon. After compression the heart fragments were weighed again. The weight loss on compression was taken as the weight of the extracellular liquid of the heart.

Determination of inulin space in hearts subjected to compression

The inulin space of the hearts was determined for direct comparison with the extracellular space measurement by compression as follows. The heart fragments were homogenised in about

8 ml. of distilled water, transferred to a 25 ml. volumetric flask and made up to the mark after the addition of 2.5 ml. of 20% metaphosphoric acid to precipitate protein. The solution was mixed, centrifuged and the inulin content of the supernatant determined. The filter papers used in the compression experiment were cut into small pieces and were then homogenised in 2% metaphosphoric acid solution. The mixture was filtered through a sintered glass filter into a 250 ml. volumetric flask. The filter was washed several times with distilled water and the washings added to the 250 ml. flask. The solution was then made up to the mark and its inulin content determined using the autoanalyzer. Addition of the inulin content of a set of filter papers to that found in the corresponding heart fragments gave the total heart inulin, and the inulin space of the heart was calculated from this as described earlier.

Chloride estimation

A modified Sendroy (1937) technique was used.

Reagents (i) solid silver iodate, B.D.H. reagent

(ii) crystalline potassium iodide, Analar grade

(iii) N/100 sodium thiosulphate solution

(iv) 1% starch solution

(v) 20% metaphosphoric acid solution

Procedure

(a) Heart sample. To a 10 ml. heart homogenate sample was added 2 ml. of metaphosphoric acid solution and approximately 40 mg. of solid silver iodate. The mixture was thoroughly shaken, filtered through Whatman No. 42 paper, and duplicate 3 ml. filtrate samples were pipetted into separate small dishes. To each of these was added a few crystals of potassium iodide and sufficient water to permit mechanical stirring. The mixture was stirred continuously and standard thiosulphate solution was added from a microburette until the iodine colouration disappeared. Starch indicator was added near the end-point.

Blank correction

To allow for the very small amount of silver iodate dissolving in the original metaphosphoric acid/heart homogenate mixture, a blank titration

was performed. This was done by putting a 10 ml. sample of doubly distilled water through the procedure outlined above.

If T = Experimental Titre using the heart homogenate sample

B = Blank Titre, then

$$\text{Corrected Titre} = T\left(1 - \frac{B^2}{T^2}\right) \text{ ml.}$$

(b) Perfusate sample. A 1 ml. portion of metaphosphoric acid solution was added to a 1 ml. perfusate sample and the mixture was diluted to 50 ml. Approximately 15 ml. of this mixture was shaken with 40 mg. of silver iodate and then filtered. A few crystals of potassium iodide were added to each of two 2 ml. aliquots of the filtrate and the mixtures were titrated with standard thiosulphate solution using the technique described above. It will be seen from the equation given above that when the experimental titre is more than 10 times the value of the blank titre, the corrected titre is bound to exceed 99% of the experimental value. When perfusate chloride estimations were performed, the dilution of the perfusate chosen was such that the correction was negligible and no blank titration needed to be performed.

Potassium determinations

The 10 ml. heart homogenate sample used for the dry weight determination was digested with 2 ml. of concentrated nitric acid for 60 hr. The digest was made up to 250 ml. The potassium concentration in this solution was now estimated in a Beckman D.U. flame photometer at 769.9 mμ. by calibrating the reading against external standards.

The author is indebted to Dr. J.W. Minnis of this department for performing these potassium determinations.

CHAPTER III

Results

General Characteristics of the Preparation

When first placed on the perfusion apparatus the hearts tended to beat strongly and irregularly. Within five min. they settled down with a strong, regular beat at a rate of approximately 220 beats/min. This rate was usually maintained throughout an experiment lasting for almost 2 hr. and it agrees well with rates found for perfused hearts by Morgan, Henderson, Regen and Park (1961).

The rate of flow of perfusate through the heart (referred to as the flow rate in future) is a factor of some importance with respect to the condition of the heart and its suitability for permeability studies. Zachariah (1960) found that with inefficient filtration of the perfusate the flow rate in a series of 11 hearts fell from an average of 8.4 ml./min./g. wet weight of heart to 5.5 ml./min.g. wet weight in 45 min. of perfusion, suggesting that some of the smaller coronary vessels were being progressively occluded. By injecting crystal violet into the heart via the cannula at the end of an experiment patches of

tissue were found not to take up the dye indicating that blockage prevented the uniform perfusion of the heart (Zachariah, 1960). With the sintered glass filter in the perfusion apparatus, no blockages of the hearts were evident after a perfusion and the flow rates fell from an average of 9.9 ml./min./g. wet weight to 6.2 ml./min./g. wet weight. In the present investigations, the flow rate was measured by clipping off the tube carrying perfusate away from the heart chamber and at the same time opening the outflow tube situated next to the cannula, so that the volume emerging into a measuring cylinder in a given time (usually 30 sec.) could be determined. With the Whatman No. 50 filter in the perfusion circuit, the flow rate of three hearts averaged 9.7 ml./min./g. wet weight at the start of an experiment and 6.7 ml./min./g. wet weight at the end of a 90 min. period. When osmium tetroxide was injected into hearts perfused in this circuit, no unperfused regions could be detected. Another consideration in connection with the flow rate is the oxygen supply of the heart. The mean oxygen consumption of the muscle was found to be 39.0 ml./g. dry weight/hr. (Williamson and Fisher, 1961), and assuming all the

oxygen is extracted from the perfusate, then the minimal flow rate which will support this requirement is 5 ml./min./g. wet weight. The flow rates found in the present investigation are well above this value so that the hearts are unlikely to become anoxic.

The basis for comparison of results

(1) Obviously, in the circumstances of these experiments, the heart wet weight is unreliable. Fisher and Williamson (1961) showed that the extracellular water per gram heart increased significantly during perfusion with a saline medium, the increase being considerable initially and smaller in later stages. But the increase was continuous throughout perfusion periods of the lengths which are used in the present work.

(2) Addis, Poo and Lew (1936) showed that the rat heart lost 4% of its protein during a two day fast. This is equivalent to less than 0.1% per hr. so that uncompensated protein catabolism would not in itself lead to significant loss of any weight material during a perfusion experiment.

(3) In the absence of endogenous nutrient, Fisher and Williamson (1961) showed that the oxygen uptake of the heart was approximately 40 µl./mg. dry

weight/hr. This is equivalent to a maximum disappearance of nutrient of approximately 16 µg./mg. dry weight/hr., i.e. a 1.6% fall in the dry weight per hr. (This is calculated on the basis of the oxidation of a $-CH_2-$unit).

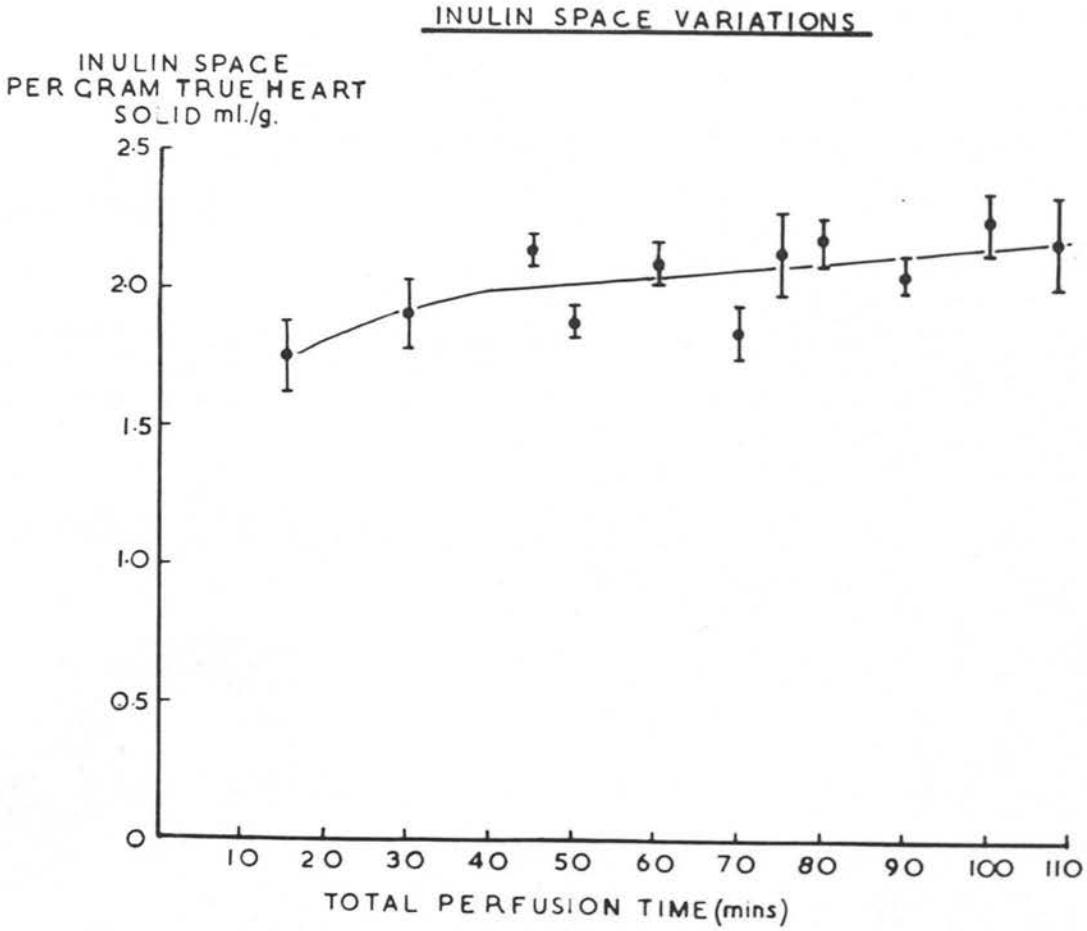
(4) It follows that, if the heart is supplied with an acceptable nutrient, such as the pyruvate used in these experiments, the dry weight may be expected to remain constant within 1% during the perfusion experiment.

In this work, therefore, measurements have been usually expressed in terms of the heart dry weight.

However, distinction must be made between the solids which are true heart material and the adventitious solids contributed by the perfusate. Thus a heart of extracellular volume x ml., perfused with a medium containing y mg./ml. of solute, will yield a dry weight differing from the true heart material by an amount xy mg. if the perfusate had completely equilibrated with the extracellular fluid. With the perfusate of the composition given in Table 9 y is 30.4 mg./ml. and since x may be taken as 0.28 ml./g. heart and the total heart solids will be approximately 200 mg./g.

heart, (Bleehen and Fisher, 1954) then the adventitious solids will be 8.5 mg., thus contributing some 4% to the total observed dry weight. This error will be larger when pentose is included in the perfusate, for each millimole will add 0.15 mg. solute/ml. perfusate. Taking such considerations to the final stage, any sugar penetrating the heart cells during a perfusion will also add to the observed dry weight. Considering, for example a perfusate containing 30mM pentose, (4.5 mg./ml.) then if the pentose equilibrated with the whole of the cell water, a further 2 mg. would be contributed to the dry weight on the basis of a heart of 1 g. with a total cell water content of 0.5 ml. Since the intracellular concentration of pentose is often as low as 10% and rarely above 70% of the extracellular concentration the solids added by intracellular pentose have been neglected in determining the true heart solids. Thus, in the results presented, the extracellular and intracellular compartments of the heart are usually expressed as a function of the true heart solids (or simply, true solids), where True Solids = Total Dry Weight - Adventitious solids, and the Adventitious solids is taken as the product xy mg. as indicated.

FIG. 23.



The points are the mean observed values ± SEM given in Table 10. The slope of the regression line through the points from 45 min. onwards does not differ significantly from zero.

Measurement of the extracellular space of the perfused heart

The suitability of inulin as an extracellular space marker was first investigated with respect to its speed of equilibration with the extracellular space. Hearts were perfused, over the intervals of time shown in Table 10, with modified Krebs bicarbonate medium containing 4mM sodium pyruvate and 2% inulin, and the inulin spaces were determined. The results given in Table 10 are shown also in Fig. 23. An increase in the volume of distribution of inulin occurs throughout the experiment. This increase is most pronounced during the first 30 min. of perfusion, and this not only illustrates the rate of equilibration of the marker, the molecular weight of which is of the order of 6000, but could also reflect a slight accumulation of water due to the perfusion pressure to which the heart is subjected. After 30 min. of perfusion, there is only a slight upward trend in the inulin space, and this is what one would expect on considering that the extensible heart tissue is subjected to a perfusion head equivalent to some 44 m.m. of mercury throughout the perfusion period. That the total tissue water

TABLE 10

Values of Inulin Space* per gram True Heart Solids
After Different Periods of Perfusion

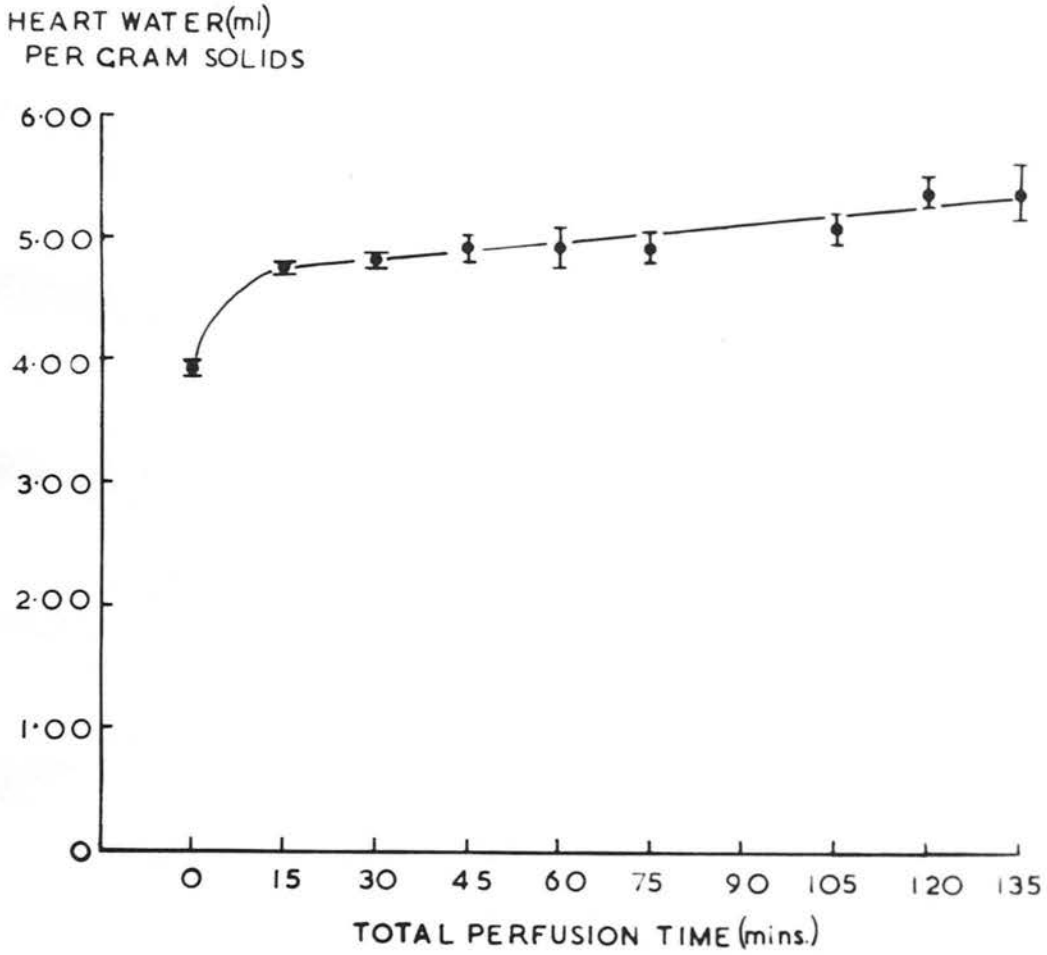
	Total Perfusion Time (min.)										
	15	30	45	50	60	70	75	80	90	100	105
Mean I.S./g. true solids (ml./g.)	1.75	1.91	2.14	1.88	2.10	1.84	2.13	2.17	2.05	2.24	2.17
SEM	0.13	0.13	0.06	0.05	0.08	0.10	0.15	0.09	0.07	0.11	0.16
Observations	5	4	12	26	33	26	5	25	27	23	7

Hearts were perfused, for the times shown, with modified Krebs bicarbonate medium containing 4 mM pyruvate and 2% inulin.

* Inulin space is abbreviated I.S.

FIG. 24.

ACCUMULATION OF WATER IN HEARTS PERFUSED OVER VARIOUS PERIODS OF TIME



The points represent the mean values, from between 3 and 14 observations, ± SEM. The straight line is the regression line of the 8 points from 15 min. onwards.

does in fact increase throughout the experiment is illustrated by Fig. 24, where the heart water is expressed as a function of the total dry weight and plotted against the perfusion time. The increase in the total heart water and the increase in the inulin space are of the same magnitude. It can be concluded that inulin equilibrates with the interstitial fluid within 30 min. and thereafter the inulin space increases due to the increase in the interstitial fluid volume.

Effect of insulin on the inulin space

Two series of hearts were perfused with a modified Krebs bicarbonate medium containing 4mM sodium pyruvate and 2% inulin for 60 min. The perfusate for one of the series contained additionally 4.0 mU.insulin/ml. The inulin space per g. true solids was determined for each heart of the experiment and the results are given in Table 11. A t-test showed that there was no statistically significant difference between the two series.

Effect of sugar on the inulin space

An experiment was performed to ensure that the addition of sugar to the perfusate did not affect the inulin space of the heart. A series

TABLE II

The Effect of Insulin on the Inulin Space of the Perfused Heart

Series	Insulin Concentration (mU./ml.)	Inulin Space per gram True Heart Solids (ml./g.)						Mean	SEM
A	0.0	1.87	1.61	1.94	1.51	1.77	1.65	1.73	0.07
B	4.0	1.83	1.70	1.67	1.91	1.62	1.85	1.76	0.02

t - Test $0.7 > P > 0.6$

Series A Hearts were perfused for 60 min. on a modified Krebs bicarbonate medium containing 4mM sodium pyruvate and 2% inulin.

Series B Perfused as for Series A but medium included 4.0 mu. insulin/ml.

of 4 hearts was perfused with a modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin for 60 min. A second series of hearts was perfused under the same conditions as the first series for 40 min. and then transferred to a similar medium containing additionally 70mM D-xylose for 20 min. The inulin spaces of both series were determined and are presented in Table 12. No significant difference between the two series was apparent.

Effect of the blotting procedure on the inulin space

An operation having a noticeable effect upon the inulin space measurement was discovered during these early experiments. When a heart has been perfused it is always halved and blotted on Whatman No. 54 paper prior to weighing and subsequent analysis. It was observed by Fisher and Young (1961) that the application of moderate pressure to the heart tissue resulted in a considerable loss of liquid, and this finding was put to good use in a method used to determine the interstitial space of the heart, (p.101). It was therefore of interest to determined if much lower pressures such as those which might be applied by over

TABLE 12

The Effect of Added Sugar on the Volume of Distribution of Inulin in the Heart

Series	Concentration of Xylose (mM)	Inulin Space per gram True Solids (ml./g.)				Mean	SEM
A	0	1.71	1.39	1.45	1.40	1.49	0.05
B	70	1.67	1.40	1.44	1.21	1.43	0.09

t - Test $0.3 > P > 0.2$

Series A Hearts perfused for 60 min. with modified Krebs bicarbonate Medium containing 4mM sodium pyruvate and 2% inulin.

Series B Hearts perfused as for Series A for 40 min. and then transferred to a similar medium containing additionally 70mM D-Xylose for 20 min.

zealous blotting, could cause any detectable effect on the measurement of the extracellular volume by the inulin space method. Two series of hearts were perfused with modified Krebs bicarbonate medium containing pyruvate and inulin for 40 min. and then transferred to a similar medium containing also 10mM D-xylose. On this perfusate for each series, one heart was perfused for each of the following times - 10, 20, 30, 40, 50 and 60 min. - these are the conditions in the main experiments of this thesis. At the end of the perfusions, one series of hearts was blotted gently after halving the tissue, while the other series was subjected to pressure during blotting. Table 13 gives the values obtained for the inulin spaces per g. true solids of the hearts. There is a statistically significant difference between the two series. In all subsequent experiments, therefore, the utmost care was taken to blot the heart tissue gently, without the application of pressure.

Comparison of extracellular space measurements by different methods

It was thought advisable to compare the value given for the extracellular space by the

TABLE 13

Comparison of Interstitial Volumes in Hearts Subjected to Different Blotting Procedures

Blotting Pressure	Inulin Space (ml.) per g. True Solids After Perfusion Times Shown						
	50 min.	60 min.	70 min.	80 min.	90 min.	100 min.	Mean & SEM
High	1.56	2.04	1.68	1.40	1.38	1.54	1.60±0.10
Low	2.90	1.71	2.31	2.32	2.03	2.38	2.28±0.16

t - Test $0.01 > P > 0.001$

All hearts were perfused with the modified Krebs bicarbonate medium containing 4 mM pyruvate and 2% inulin for 40 min. before transfer to a similar medium, containing also 10mM D-Xylose, for 10, 20, 30, 40, 50 or 60 min.

inulin space, with the values given by the raffinose space and by the compression method developed by Fisher and Young (1961).

Comparison of Inulin and Raffinose spaces

Two series of hearts were perfused for 60min. with modified Krebs bicarbonate media containing either 2% inulin (Series A) or 10mM raffinose (series B), and the volumes of distribution of the extracellular markers determined. The results are given in Table 14, where the spaces are expressed as a function of the wet weight of the heart. There is no statistically significant difference between the two series.

Comparison of inulin space and weight loss by compression

Each of a series of 12 hearts was perfused for 30 min. on a modified Krebs bicarbonate medium containing 2% inulin and 4mM pyruvate. At the end of the perfusions the hearts were halved, blotted gently and weighed. The halves of each heart were then compressed in the apparatus shown in Fig. 22 as described under Methods, (p. 102) and the weight loss due to compression determined. The inulin spaces of the hearts were also deter-

TABLE 14

Comparison of Inulin and Raffinose Spaces of Hearts Perfused for 60 min.

	Tissue Space (µl./g. Heart)	
	A Inulin	B Raffinose
	387	421
	406	376
	347	366
	279	355
	299	305
	308	285
	313	401
	295	379
	319	351
	336	344
	317	319
	299	305
	336	265
Mean	326	344
SEM	10	13

t - Test $0.3 > P > 0.2$

Series A - Perfusate - modified Krebs bicarbonate medium containing 4mM sodium pyruvate and 2% inulin.

Series B - Perfusate - modified Krebs bicarbonate medium containing 10mM raffinose.

mined as described on p. 103. Table 15 gives the results of this experiment. There is no significant difference between the interstitial volumes yielded by the two methods.

Cell water in perfused hearts

Fisher and Zachariah (1961) found their measurements of the intracellular water (ICW) per g. dry weight of the heart to give such consistent results that they eventually relied upon the relationship to calculate the intracellular water of the heart directly from the dry weight, no interstitial volume determination being undertaken. The ICW/g. solids values were not affected by the duration of the perfusion experiment or by the addition of insulin to the perfusion medium. It is as well to mention that only one perfusate sugar concentration was used throughout the experiments of Fisher and Zachariah. In the early experiments of the present work it became evident that the values of ICW/g. dry weight for hearts differed considerably (see Table 16) from the value of 2.45 ml./g. given by the workers mentioned above. The coefficient of variation of the values for ICW/g. solids in a group of 32 hearts studied by

TABLE 15

Comparison of Inulin and Compression Spaces of Hearts

	Tissue Space (µl./g. Heart)	
	Inulin	Compression
	338	373
	345	274
	376	368
	298	354
	360	346
	318	397
	342	253
	339	289
	339	166
	326	347
	345	307
	345	351
Mean	339	319
SEM	6	19

t - Test $0.4 > P > 0.3$

All hearts were perfused for 30 min. on a modified Krebs bicarbonate medium containing 2% inulin. The weight losses on compression of the hearts and the inulin spaces, were determined as described under 'methods'.

Zachariah (1960) is 5.31% while the coefficient of variation for values obtained from a group of 42 rats studied in the experiment mentioned below is 6.82%. It was felt that the variance of the values of ICW/g. solids was too high to merit direct calculation of the cell water from the heart dry weight. Experiments were undertaken to determine the factors influencing the intracellular water of perfused hearts in the present work. It was hoped that the sources of variation might be better controlled and the values of ICW/g. true solids taken as indications of the general state of the hearts used. In these experiments the cell water is expressed as a function of the true heart solids, rather than the total solids as used by Zachariah (1960).

Day to day variations in cell water values

This was investigated by examining the data obtained from an experiment conducted as follows. Hearts were preperfused for 40 min. with a modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin and then transferred to a similar medium containing 10mM L-arabinose. The hearts were perfused with this second medium for 10, 20, 30, 40, 50 or 60min. The experiment employed

42 hearts over a period of 7 days, and on any one day 6 hearts were perfused, one at each of the perfusion times given above. The values for the intracellular water were found by subtracting the inulin space of each heart from the total water content of that heart. The values for ICW/g. true solids were then computed and are given in Table 16. The analysis of variance is given in Table 17. Day to day variations do significantly affect the intracellular water/g. true solids values.

Effect of changes in perfusate sugar concentrations on the cell water

The variability found in groups of values of ICW/g. solids is hardly surprising when one considers the osmotic effects operating in the perfused preparation. The basic perfusate, referred to as the modified Krebs bicarbonate medium, given in Table 9 is approximately isosmotic with the cardiac cell water when it contains 4mM pyruvate. Addition of pentose to this perfusate results, therefore, in a degree of hypertonicity depending upon the sugar concentration, and corresponding osmotic forces will be exerted upon the cells of the heart. Therefore, hearts perfused

TABLE 16

Values of Intracellular Water/g. True Solids in Seven Series of Hearts Perfused on Different Days

	Intracellular Water per gram True Heart Solids (ml./g.) at the Total Perfusion Times Shown (min.)						Mean Daily Value
	50	60	70	80	90	100	
Day 1	2.92	2.75	2.94	3.15	3.11	2.96	2.97
Day 2	2.99	2.86	2.98	3.15	2.87	3.13	3.00
Day 3	2.72	2.99	2.67	2.89	2.76	2.99	2.84
Day 4	2.66	2.92	2.87	3.13	2.93	2.93	2.91
Day 5	2.79	2.90	2.83	2.97	2.84	3.08	2.90
Day 6	2.98	2.87	3.00	3.06	3.19	3.19	3.05
Day 7	3.38	3.77	2.85	3.33	2.85	3.55	3.29
Mean	2.92	3.01	2.88	3.10	2.94	3.12	

Hearts were perfused with modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin for 40 min. and then transferred to a similar medium containing 10mM L-Arabinose for the times given.

TABLE 17

Analysis of Variance of Data on Intracellular Water per gram True Solids Presented in Table 16

Source of Variation	Degrees Freedom	Sums of Squares	Mean Square	Variance Ratio	Probability
Total	41	20084.6			
Times	5	3422.3	684.5	2.33	$0.2 > P > 0.05$
Days	6	7859.2	1309.9	4.46	$0.01 > P > 0.001$
Residual	30	8803.1	239.4		

with a medium containing a high sugar concentration would be expected to reveal a larger withdrawal of water from the cells than hearts exposed for the same period of time to media containing low concentrations of sugar. It was possible with one set of data to test this prediction. The hearts were perfused on a sugar-free modified Krebs bicarbonate medium with inulin and pyruvate for 30 min. and then transferred to perfusates containing four different concentrations of sugar. The duration of the perfusion with a sugar-containing medium was 15, 30 or 45 min. The entire experiment took a number of weeks, and on any one day all four sugar concentrations were used so that day to day variations would not interfere with the investigation. The results for the ICW/g. true solids determined in these hearts is given in Table 18. Table 19 gives an analysis of variance of the data. It will be seen that the external sugar concentration does indeed exert a statistically significant effect upon the ICW/g. true solids and the general trend is as one would expect - smaller cell water volumes being found in the presence of high perfusate sugar concentrations.

TABLE 18

Intracellular Water per gram True Solids (ml./g.) for Hearts Perfused with Various Concentrations of L-Arabinose for Different Times

Perfusion Time(min.)	External Sugar Concentration (mM) 10	15	20	30	Mean ± SEM
15	3.13	2.92	3.08	2.96	
	2.96	2.85	2.76	2.76	2.975
	2.90	3.11	3.00	2.75	±0.045
	2.92	3.35	3.30	2.85	
Mean	2.97	3.06	3.04	2.83	
30	2.92	2.89	2.71	2.75	
	3.06	3.06	3.04	2.82	2.987
	3.29	3.18	3.19	2.92	±0.040
	3.11	3.01	2.96	2.88	
Mean	3.10	3.04	2.98	2.84	
45	3.13	2.99	2.89	2.95	
	3.07	3.04	2.92	2.93	2.999
	3.26	3.13	2.91	3.01	±0.037
	3.03	3.01	2.89	2.83	
Mean	3.12	3.03	2.90	2.93	
Overall	3.065	3.045	2.971	2.867	The co-efficient of Variation of the 48 values is 5.0%
Mean ± SEM	±0.037	±0.039	±0.048	±0.026	

The hearts were perfused with modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin for 30 min. and then transferred to similar media containing, the concentrations of Arabinose shown above, for 15, 30 or 45 min.

TABLE 19

Analysis of Variance of the Intracellular Water per gram True Solids Results Shown in Table 18

Source of Variation	Degrees Freedom	Sums of Squares	Mean Square	Variance Ratio	Probability
Total	47	10661.9			
Times	2	47.5	23.8	0.13	
Concentrations	3	2878.7	959.6	5.15	$0.01 > P > 0.001$
Interaction	6	1027.2	171.2	0.92	
Residual	36	6708.5	186.3		

The effect of the duration of perfusion with sugar on the cell water

Fisher and Zachariah (1961) showed that when a heart is perfused with a medium containing sugar the penetration of sugar into the cells of the heart increases with the duration of perfusion, and experiments supporting this observation will be presented in a later section. This being so, as the duration of perfusion increases, water should be drawn into the cardiac muscle cells as the number of osmotically active molecules in the cells is increased by addition of sugar. This prediction was tested by examining the values for ICW/g. true solids obtained for hearts perfused for 40 min. with a modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin and then transferred to a pentose-containing medium for perfusion over the range of times shown in Table 20. This data was obtained over a period of several months, and since the factor under examination was the perfusion time, on any one day, all 6 perfusion times were studied so that day to day variations would not interfere with the investigation. It will be seen that insulin was used in some of the experiments and a study of its effect

TABLE 20

Mean Intracellular Water per gram True Solids (ml./g.)

Under Various Conditions

The number of hearts giving each mean value is given in parenthesis

Sugar	Conc. (mM.)	Insulin (mU./ml.)	Duration of Perfusion with Sugar (min.)					
			10	20	30	40	50	60
Xylose	9.65	0	2.78 (8)	2.85 (8)	2.80 (7)	2.94 (7)	2.94 (8)	2.96 (6)
Arabinose	9.25	0	2.92 (7)	2.99 (8)	2.88 (8)	3.14 (8)	2.94 (8)	3.07 (8)
Xylose	54.40	1.0	2.90 (5)	3.00 (5)	3.04 (5)	3.22 (5)	3.13 (5)	3.24 (4)
Arabinose	30.00	1.0	3.10 (6)	3.01 (6)	3.15 (6)	3.18 (5)	3.16 (6)	3.11 (5)
Overall Means			2.93	2.96	2.97	3.12	3.04	3.10

The perfusion media were modified Krebs bicarbonate media containing 4mM pyruvate, 2% inulin and either xylose or arabinose in one of the concentrations shown above. The preperfusion, in the absence of sugar, was for 40 min.

is considered in the analysis of variance of the data given in Table 21, but this aspect will be dealt with later. The analysis of variance shows the effect of perfusion time to be statistically significant. There is a slight increase in the overall mean ICW/g. true solids values as the perfusion time increases, and this was predicted on the basis of the osmotic effects.

Correction of Cell water data for the osmotic effects

The effects on the cell water of the osmotic forces operating in the perfused heart can be expressed quantitatively as follows:-

If m = milliosmolarity of modified Krebs bicarbonate medium plus 4mM sodium pyruvate = 291 milliosmols.

i = milliosmolarity of extracellular indicator (2% inulin) = 4 milliosmols.

x = milliosmolarity of sugar in medium

f = fractional penetration of cell water by sugar in time t i.e. the intracellular concentration at time t expressed as a fraction of the medium concentration,

then at time t, the effective external milliosmolarity = m+x+i milliosmols. Intracellular milliosmolarity = m + fx milliosmols.

TABLE 21

Analysis of Variance of Data on Intracellular Water per gram True Solids Given in Table 20

Source of Variation	Degrees Freedom	Sums of Squares	Mean Square	Variance Ratio	Probability
Total	23	3974.6			
Times	5	1248.4	249.7	5.64	$0.01 > P > 0.001$
Sugars	1	301.0	301.0	6.80	$0.05 > P > 0.01$
Insulin	1	1717.0	1717.0	38.79	$0.001 > P$
Residual	16	708.2	44.3		

Thus the intracellular water will be smaller than its normal value by a factor $\frac{m + fx}{m + x + i}$. Consequently, multiplication of the observed intracellular water/g. true solids by the factor $\frac{m + x + i}{m + fx}$ will give the true, or equilibrium cell water/g. true solids (ICWo/g.). This factor will be referred to as the osmotic correction.

A test of the validity of these considerations can be made by correcting the values of ICW/g. true solids given in Table 20. Since the effect of the duration of perfusion time on the cell water has been assumed to be due to the osmotic effects dealt with above, then correction of the cell water values for such effects should eliminate the perfusion time as a statistically significant factor affecting the values. The mean values of f corresponding to the mean values of ICW/g. true solids of Table 20 will be presented in a later section, but it is nevertheless convenient to give the results of applying the osmotic correction at this juncture. The results are shown in Table 22 and the analysis of variance is shown in Table 23. The perfusion time now has no statistically significant effect upon the ICW/g. true solids. The validity of the osmotic correc-

TABLE 22

Mean Osmotically Corrected Intracellular Water per gram True Solids (ml./g.) Under Various Conditions

Sugar	Conc. (mM)	Insulin (mU/ml.)	Duration of Perfusion with Sugar (min.) (and with insulin where indicated)					
			10	20	30	40	50	60
Xylose	9.65	0	2.89 (8)	2.96 (8)	2.90 (7)	3.04 (7)	3.04 (8)	3.06 (6)
Arabinose	9.25	0	3.04 (7)	3.10 (8)	2.98 (8)	3.25 (8)	3.04 (8)	3.17 (8)
Xylose	54.40	1.0	3.35 (5)	3.38 (5)	3.33 (5)	3.53 (5)	3.39 (5)	3.50 (4)
Arabinose	30.00	1.0	3.39 (6)	3.26 (6)	3.39 (6)	3.36 (5)	3.34 (6)	3.27 (5)
Overall Means			3.17	3.18	3.15	3.29	3.20	3.25

The values were obtained by applying the osmotic correction to the values for intracellular water per gram true solids given in Table 20.

TABLE 25

Analysis of Variance of Data on Osmotically Corrected Intracellular Water per gram True Solids Given in Table 14

Source of Variation	Degrees Freedom	Sums of Squares	Mean Square	Variance Ratio	Probability
Total	23	8564.0			
Times	5	626.0	125.2	1.65	$P > 0.2$
Sugars	1	22.4	22.4	0.28	$P > 0.2$
Insulin	1	6700.4	6700.4	88.16	$0.001 > P$
Residual	16	1215.2	76.0		

tion can be similarly tested by considering the data of Tables 18 and 19 where the perfusate sugar concentration was shown to significantly alter the ICW/g. true solids. The osmotically corrected values derived from those of Table 18 are presented in Table 24. The analysis of variance of the osmotically corrected data (Table 25) shows the effect of the perfusate sugar concentration now not to be significant. The coefficient of variation of the 48 osmotically corrected values is 4.4% and this is very close to the values of 5.0% obtained for the uncorrected data.

Insulin and cell water

Referring back to the data of Table 20 giving the ICW/g. true solids for hearts perfused in the presence of different concentrations of sugar and insulin, and to Table 21 which gives the analysis of variance of that data, it will be seen that insulin apparently has a quite striking effect upon the values. In view of the osmotic effects mentioned this effect of insulin could simply reflect the osmotic retention of water associated with higher penetrations of the cell water by sugar when the hormone is present.

TABLE 24

Osmotically Corrected Intracellular Water per gram True Solids (ml./g.) For Hearts Perfused with Different Concentrations of L-Arabinose For Different Times (derived from Table 18)

Perfusion Time	Perfusate Sugar Concentration (mM) 10	15	20	30	Mean ± SEM
15 min.	3.25	3.08	3.26	3.27	
	3.05	3.00	2.93	3.06	3.17
	3.02	3.29	3.20	2.99	±0.05
	3.04	3.51	3.56	3.15	
30 min.	3.01	3.03	2.87	2.99	
	3.18	3.19	3.22	3.08	3.16
	3.39	3.34	3.38	3.17	±0.04
	3.23	3.16	3.16	3.19	
45 min.	3.24	3.13	3.07	3.20	
	3.16	3.14	3.06	3.14	3.16
	3.37	3.27	3.10	3.28	±0.02
	3.13	3.12	3.06	3.09	
Mean	3.17	3.19	3.16	3.13	
SEM	±0.04	±0.04	±0.05	±0.03	

The coefficient of variation of the 48 values is 4.4%

The hearts were perfused with modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin for 30 min. and then transferred to a similar medium, containing one of the concentrations of arabinose shown above, for 15, 30 or 45 min.

TABLE 25

Analysis of Variance of the Values of Osmotically Corrected Intracellular Water per gram True Solids Given in Table 24

Source of Variation	Degrees Freedom	Sums of Squares	Mean Square	Variance Ratio	Probability
Total	47	9235.5			
Times	2	3.3	1.7	0.01	
Concentrations	3	193.8	64.6	0.35	$P > 0.2$
Interaction	6	1337.3	190.0	1.04	
Residual	36	7701.1	183.4		

That this is not the case, however, is indicated by the data of Tables 22 and 23. The insulin effect is still significant after applying the osmotic correction to allow for the penetration of pentose into the heart cells. The possibility that an entirely new effect of insulin had come to light merited investigation. Since the data given in Tables 20 and 22 had been obtained over a period of months it was possible that the difference between the insulin and non-insulin data was due to alterations in seasonal factors as yet uncontrolled. A direct experiment was therefore necessary to establish unequivocally any dependence of the cell water on the perfusate insulin concentration. Two series of hearts were perfused on the same day, for 60 min. with a modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin. For one of these series, insulin was included in the perfusate in a final concentration of 4.0 mu./ml. Since any effect of insulin on the cell water is most likely to be mediated by an alteration in the electrolyte distribution in the heart, estimations of cell chloride and potassium levels, as well as the cell water, were included in this experiment. The

results are given in Table 26. Where the intracellular water is concerned, there is no significant difference between the insulin-treated and the control hearts, and in fact, there is a slight tendency for the control values to be the larger ones. These considerations of an apparent insulin effect on the cell water of the heart emphasise again the influence of day to day variations on the results.

The values for the percentage penetration of the intracellular water by chloride and for the intracellular potassium concentration in the presence and absence of insulin show that while the two chloride distribution series do not differ significantly, there is a small but real increase in the cell potassium concentration when insulin is added. This is an interesting finding in view of the results of other workers (Creese, 1954; Zierler, 1957) and it will be discussed later.

Permeability changes in the perfused heart

The replacement in the perfusion apparatus of the sintered glass filter by a Whatman No. 50 filter paper (p. 94) made it necessary to re-investigate the changes in sugar permeability in the perfused heart during long periods of perfusion.

TABLE 26

Effect of Insulin on Potassium Chloride and Water Content of Cells of the Perfused Heart

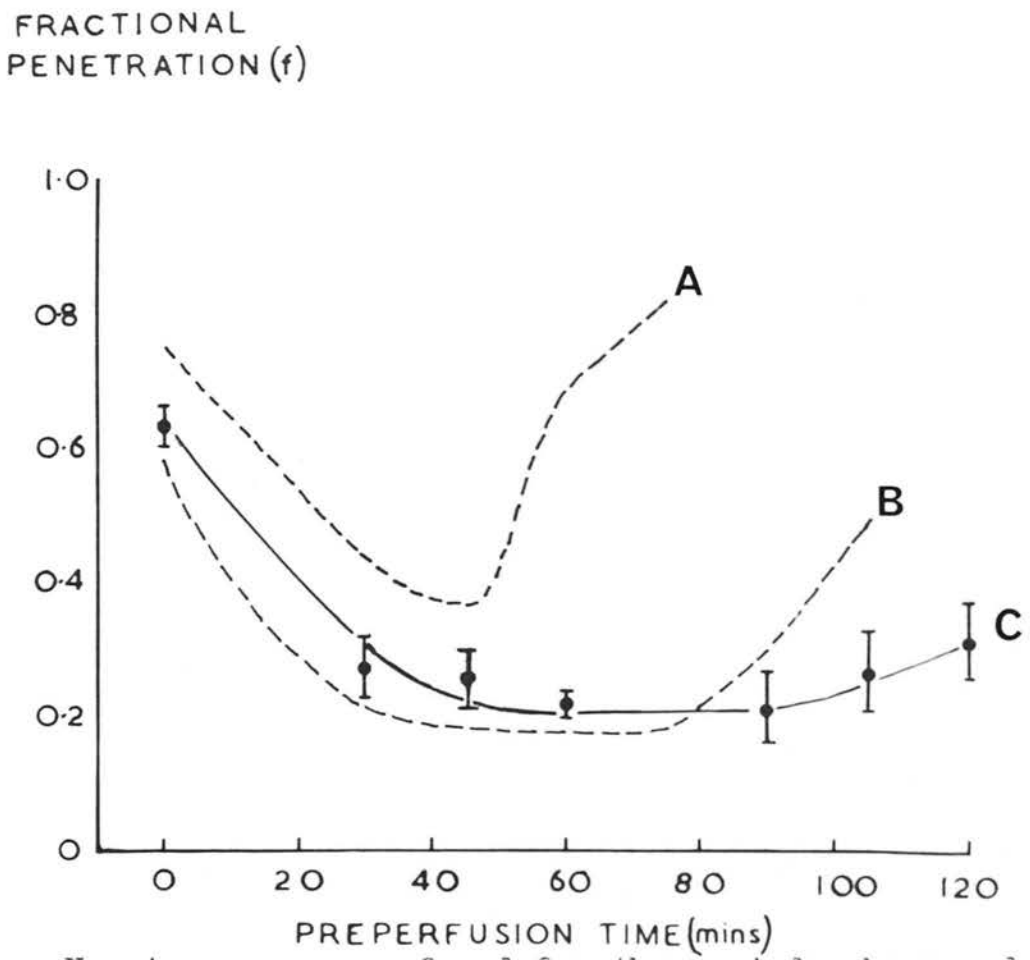
	Osmotically Corrected ICW/ g. True Solids (ml./g.)		Intracellular* K^+ Concentration (m.Eq./l.)		Percentage Penetration of Cell Water by Chloride	
	Control	Insulin	Control	Insulin	Control	Insulin
	3.06	3.04	124.4	161.1	22.4	18.2
	3.00	2.98	154.2	156.8	21.8	22.8
	3.08	2.90	141.0	147.4	16.6	18.8
	2.82	2.65	135.9	146.3	24.4	27.7
	2.89	2.94	138.3	155.6	27.5	27.3
	3.10	2.87	133.3	141.4	30.1	26.5
	2.38	2.62	160.1	167.7	25.3	27.6
	3.10	2.58	133.8	155.3	30.8	16.8
Mean	2.93	2.82	140.1	154.0	24.9	23.2
SEM	0.09	0.06	4.13	3.02	1.66	1.65
t Test	$0.2 > P > 0.1$		$0.01 > P > 0.001$		$0.4 > P > 0.3$	

Both 'control' and 'insulin' series were perfused with a modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin. No pentose was present. In the insulin series, the final concentration of insulin was 4.0 mu./ml. perfusate. The perfusion time was 60 min.

*The potassium concentrations are expressed in terms of the actual cell water determined experimentally.

FIG. 25.

CHANGES IN PERMEABILITY TO L-ARABINOSE



Hearts were preperfused for the periods shown and then perfused with 30mM L-Arabinose for 15 min.

Curves 'A' and 'B' were obtained by Zachariah (1961) using the sintered glass filter.

'A' - modified Krebs bicarbonate medium
'B' - modified Krebs bicarbonate medium containing 4mM pyruvate and 4 ml. dialyzed ox-serum/100 ml.

Curve 'C' was obtained in the present work using a paper filter. The medium is the modified Krebs bicarbonate solution containing 4mM pyruvate. The points are the mean observed values ± SEM as given in Table 27.

Hearts were preperfused for various intervals of time on a modified Krebs bicarbonate medium containing 4mM sodium pyruvate and 2% inulin, and then transferred to a similar perfusate containing 30 mM L-arabinose for 15 min. in every case exactly as in the experiments of Zachariah (1961). The percentage penetrations of cell water by the sugar were determined as described in Chapter I of this section, and the results are presented in Table 27. Fig. 25 gives a plot of the results and also includes the curves obtained by Zachariah (1960) using the sintered glass filter. Thus 'A' was obtained using a modified Krebs bicarbonate medium containing 10mM raffinose to act as extracellular marker and 'B' was obtained using a similar medium to which 4mM sodium pyruvate and 4 ml. of dialysed ox serum/100 ml. perfusate had been added. The perfusate used in the present work giving curve 'C' differs from that of 'B' only in that no ox serum is present. It will be seen that a rather longer period of stable permeability properties is achieved by perfusing hearts with the medium used in the present work.

TABLE 27

Changes in Permeability of Perfused Heart to L-Arabinose

	Duration of Preperfusion (min.)						
	0	30	45	60	90	105	120
Percentage Penetration of cell water	71.0	26.4	17.9	15.8	27.1	15.4	34.7
	60.6	11.0	31.1	24.8	25.4	52.2	9.9
	54.7	21.9	17.9	29.6	36.5	41.6	31.9
	60.9	29.9	17.6	17.3	15.4	17.0	49.2
	70.3	45.8	33.5	25.0	4.2	14.9	20.3
	-	29.7	-	-	-	21.8	42.3
Mean	63.5	27.5	23.6	22.5	21.7	27.1	31.4
SEM	3.1	4.7	3.6	2.6	5.5	6.5	5.9

Hearts were preperfused on a modified Krebs bicarbonate medium containing 4mM pyruvate and 2% inulin for the times shown, and then transferred to a similar medium, containing 30mM L-Arabinose, for 15 min.

Where there is a dash, the percentage penetration result was considered unacceptable because of some defect in the perfusion or analytical techniques.

CHAPTER IV

Discussion

Equilibration of inulin with the interstitial fluid of the heart

A major portion of this section has been devoted to studies on the use of inulin as a marker of the interstitial space in the perfused heart, for the measurement of this space must be entirely reliable if valid sugar-uptake experiments are to be performed.

Inulin has been employed as a marker by several groups of workers, some using the whole animal (Wilde, 1945; Levitt and Gaudina, 1950) and others using the isolated preparation, (Boyle, Conway, Kane and O'Reilly, 1941), but in spite of its value in these experiments, its suitability has been questioned by Cotlove (1954). He infused inulin into intact rats and found that the curve of its distribution was of a biphasic nature the second and slower portion extending over some 15 hr. and the inulin space finally corresponded in magnitude to the thiosulphate and sorbitol spaces. However, as Coxon and Robinson (1959) have shown in connection with the equilibration of C^{14}-labelled bicarbonate, there is a great variation in the speed of equilibration with

different tissues with even the most readily diffusible plasma solutes. This may well be due in great part to the enormous variation in the blood flow to different tissues. In skeletal muscle, the largest single tissue in the body, the resting blood flow is in the region of 5 ml. per 100 g. wet weight of tissue per min., corresponding to a total of about 1500 ml./min. By comparison, although the blood flow through the two human kidneys is also some 1500 ml./min., the weight of the organs is only 250 g. so that the flow is 600 ml./100 g./min. Where the heart is concerned, the coronary blood flow is of the order of 50 ml./100 g./min. and this is ten times the flow through the skeletal muscle. Thus the work of Cotlove on the change in the volume of distribution of inulin during infusion of the compound into intact rats cannot be taken as evidence against the use of inulin as an extracellular space marker.

In the present work, it has been demonstrated that the curve obtained for the change in the inulin space with the duration of the perfusion of the heart (Fig. 23) increases only slowly after the first 30 min.

This increase is paralleled by an increase in the total water of the heart (Fig. 24) and this is only to be expected since the heart is subjected to a perfusion head equivalent to 44m.m. of mercury throughout an experiment. Thus the inulin space, which can be taken as being the same as the extracellular space after 30 min. of perfusion, increases after this time as the small amount of oedema of the heart increases. It should be noted that the shortest total perfusion time for which measurements of sugar-uptake are made is 40 min. in later experiments.

In considering the speed of equilibration of inulin with the interstitial fluid, it is interesting to note that the molecular weight of the fructosan can be taken as 5000 - 6000, and this is close to the accepted molecular weight of the monomeric form of insulin. It would consequently be expected that the rates of diffusion of inulin and insulin through the extracellular compartment of the heart would be comparable. Thus, any experiment involving a study of the effects of insulin on the cells of the heart should take into account this diffusion process and in particular, when sugar uptake is studied, the perfusion

with a sugar-containing medium should be started only after a 30 min. perfusion period with the insulin-containing medium.

Comparison of inulin space with extracellular space values given by other methods

It is pertinent now to consider the values given for the extracellular space of the heart by other workers. Lemely and Meneely (1952) using the sodium space, give a mean value of 28% of the heart. Bleehen and Fisher (1954) obtained a mean value for the inulin space of 28% of the heart, and Young (1960) using the weight loss on compression as the extracellular space method, obtained data giving a mean value of 33% of the heart. Morgan, Henderson, Regen and Park (1961) found a value of 36% using the sorbitol space method. The difference of some 20% between the result of Bleehen and Fisher using inulin and of Morgan et al. using sorbitol seems to constitute a serious discrepancy in extracellular space measurements for one type of preparation. However, it should be noted that comparatively slight changes in the conditions of the experiments in which the extracellular space

values are estimated can drastically effect the results. This is perhaps best illustrated by reference to the results obtained in the present work showing the effect of variations in the blotting procedure on the inulin space of the heart. Referring back to Table 13, taking the intracellular water of the heart per g. true solids as 3 ml./g., the mean values found for the inulin space are 37% of the heart with the gentle blotting procedure and 29% if the blotting pressure is high. The difference is similar to that found between the results of Bleehen and Fisher and Morgan et al. and it involves only a small change in technique whilst using the same compound as the extracellular marker.

It is nevertheless relevant at this point to compare the values given for the extracellular space by the inulin space method with the values given by other methods under the experimental conditions used here. The inulin space has been compared with the raffinose space (Table 14) on the one hand and with the weight loss by compression on the other (Table 15). There is no significant difference between the inulin space values

and the values given by either of the other methods. The mean inulin space given by the 25 hearts used in these experiments is 33% of the heart, and this is near the middle of the results given by the other workers mentioned earlier. The addition of sugar (Table 12) and of insulin (Table 11) to the perfusate has no effect upon the inulin space and in view of all the considerations made here, it was felt that the use of inulin as an extracellular marker was justified in the present work.

Variability of measurements of extracellular and intracellular spaces of the heart

With regard to the extracellular space measurements the coefficient of variation calculated for 42 hearts perfused with 9.25 mM L-arabinose in the absence of insulin is 18.7%. This high variability will be due largely to the difficulty of blotting the hearts to the same extent. With this high variability it is clearly advisable to estimate the extracellular space for individual hearts rather than to determine a mean value from one particular series and to use this throughout other experiments. It is felt, therefore, that

the method used by the workers of Park's laboratory, (Morgan et al. 1961) in which the mean sorbitol space given by 28 rats is taken as the extracellular value in other experiments, is subject to error. The sugar-uptake measurements will also then be subject to error. It can be calculated from the data of Morgan et al. that the coefficient of variation of their sorbitol space values used to yield the mean value of 36% is approximately 15% so that the variability is similar to that found in the present work.

On the other hand, for the series of hearts which gave a coefficient of variation of 18.7% for the extracellular space values, the coefficient for the intracellular space values per g. true solids is 6.8%. This figure is similar to that which can be calculated from Zachariah's data (1960), the result from 32 hearts being 5.3%. In view of the low variability of these values Fisher and Zachariah (1961) used the mean figure of 2.45 ml./g. for the intracellular water/g. solids obtained from the 32 hearts as the value from which to calculate the cell water of hearts used in other experiments directly from the dry weights.

It will be seen in the final section of this thesis that a small change in the sugar uptake estimate for just one heart in a series of 30 - 40 can alter the values calculated for the parameters of the transport process. The precision of the measurement of the intracellular space is a major factor affecting the reliability of the measurement of sugar-uptake by the heart cells. It was felt, therefore, that the variability shown in the values for intracellular water/g. true solids is too high to merit the use of a predetermined mean value to calculate the cell water directly from the heart dry weight. This is a source of variability in the results of Fisher and Zachariah (1961) which can be cut down, and in the present work, the individual cell water values of hearts have always been determined from the results of the individual total water and extracellular water values.

Cell water values in perfused hearts

The comparatively low variability of the values for the intracellular water/g. true solids is a reassuring finding, not only because it compares favourably with that of Zachariah, but also

since the values themselves are a measure of the reliability of certain techniques. Thus the cell water determinations are dependent upon the inulin extraction and estimation techniques, upon the correction made for the endogenous inulin-like materials of the heart and upon the weighings involved. It is interesting therefore to compare the values obtained here with those of other workers. There is a large discrepancy, for instance, between the overall mean value of 2.45 ml./g. for the ICW/g. dry weight found by Zachariah, a value apparently independent of the sugar penetration of the cells in the presence and absence of insulin, and any of the values found in the present work, (Tables, 16, 18 and 20). However, the figures of these tables refer to the true heart solids, so that for a fair comparison, the adventitious solids of Zachariah's perfusate should be considered. These include 4mM pyruvate 10mM raffinose, 30mM pentose and an amount of dialysed ox-serum giving a final concentration of protein of 2 m.g/ml. Adding the solids contributed by these to the basic perfusate solids of 9.96 mg./ml and the total is 22.84 mg./l. Taking the interstitial volume and dry weight of

hearts from Zachariah's data (1960) as 0.35 ml./g. and 188 mg. respectively, then on correcting for the adventitious solids, the ICW/g. true solids becomes 2.57 instead of 2.45 ml./g. This is still considerably lower than the value of 3.02 ml./g. given by 358 rat hearts in the present work. (This value has been calculated from the values given in Tables 16, 18 and 20 and also from results from experiments mentioned in later sections). The results of other workers show better agreement. Lowry, Hastings, Hull and Brown (1942) obtained a value of 2.51 for the intracellular water of hearts per g. fat-free solids. Recorrecting for the fat content of the heart, taking this to be 16 mg./g. heart (Handbook of Biolog. Data. Spector, 1956) the result becomes 2.35 ml./g. Other corrections, for instance to allow for the blood contained in the hearts, had been made by the workers themselves. However, in these studies, after the hearts had been excised from the rats, trimmed free of auricles, blotted free of blood and finely minced, the extracellular compartment was calculated on the basis that the chloride ion was confined to

this compartment. This assumption is valid for hearts treated as described in the experiments of Lowry et al., but would not be valid for 'in vitro' preparations (Fenn, Cobb and Marsh, 1934; Eggleton, Eggleton and Hamilton, 1937; Boyle, Conway, Kane and O'Reilly, 1941). Zachariah (1961) using the perfused heart has shown that the chloride ion penetrates the cell water until its concentration in the cell is 23% of its perfusate concentration, whilst the data obtained in the present work give a value of 25%. Assuming that the chloride ions entering the cells are accompanied by monovalent cations, then due to the osmotic effects, the intracellular water estimated in the hearts in the present work is likely to be larger than the value 'in vivo' by a factor of 1.25. The application of this correction factor brings the mean value for ICW/g. true solids in the present work from 3.02 ml./g. to 2.42 ml./g.

From the data given by Lowry et al. for a series of Yale Cornell rats, it can be calculated after allowing for the solids contributed by the heart fat that the mean value for the ICW/g. true solids is 2.44 ml./g.

Referring now to the data of Lemley and Meneely (1952) the mean ICW/g. for 11 Sprague Dawley rats is 2.07 ml./g., the mean total solids being 234.3 mg./g. heart and the mean intracellular water being 485.4 µl./heart. In these experiments, the hearts had merely been blotted free of surface blood, so that blood solids in the vasculature are included in the

total dry weight. Taking the total blood solids as 183.8 mg./ml. (Biochemists Handbook, 1961) and assuming that a heart of 1 g. has vasculature containing 0.05 ml. of blood, then the adventitious blood solids contribute 9.2 mg. to the dry weight. Adding 2 mg. to this to allow for interstitial solids, and a total of 11.2 mg. of adventitious solids is contributed to the dry weight. The true heart solids is therefore nearer 223.1 mg. and the ICW/g. true solids is then 2.19 ml./g. ~~[Correction for the chloride space method of interstitial volume measurement brings this to 2.00 ml./g.]~~ From the work of Lowry et al. and of Lemley and Meneely, three mean values of the intracellular water per g. true heart solids have been obtained. These are ~~3.09~~ 2.33 ml./g for Wistar rats, ~~3.04~~ 2.44 ml./g. for Yale Cornell rats, and ~~3.06~~ 2.19 ml./g. for Sprague Dawley rats. These values should not be directly compared, however, with the value of 3.02 ml./g. obtained with Wistar rats in the present work ~~[and which was used for comparison with Zacharish's data.]~~ The value of ~~3.02~~ 2.42 ml./g. does not take into account any osmotic withdrawal of water from the heart cells due to perfusion of the heart with a medium made hypertonic by the

presence of sugar. [~~Those osmotic forces should be similar in Zacharish's preparation and the one used here.~~] The three mean values obtained from the data of Lowry et al. and of Lemley and Meneely were obtained from measurements made in the absence of such osmotic forces, and should be compared therefore with the mean value calculated from 2·42 ~~3·02~~ ml./g. by applying the osmotic correction. The mean value so obtained for the 358 hearts is 2·56 ~~3·20~~ ml./g., and this is not very different from the three values calculated from the data of the other workers, especially when one considers the variability shown by the ICW/g. true solids measurements in the present work.

The agreement of the cell water results with those of other workers is a reassuring finding, for one might expect that normal cardiac muscle cells have reasonably constant composition. Assuming these cells act as perfect osmometers, then a further test of the soundness of the perfusion technique and of the method of determining the cell water can be made by applying the osmotic correction. Thus, if the cell water values alter in a manner which is predictable on the basis of the particular degree of hypertonicity of the perfusate

which depends upon the pentose concentration, then the overall experimental procedure would appear to be sound. This is, in fact, seen to be the case, for on applying the osmotic correction factor, it was possible to nullify the effect on the cell water, of the sugar outside and inside the heart cells over a wide range of perfusate sugar concentrations.

The changes induced in the cell water by the sugar content of the medium perfusing the heart, and by the entry of sugar into the heart cells, are particularly relevant to the main problem of this thesis. The experiments described in the final section involve the determination of the fractional penetration by sugars of the cell water of the heart at different times of perfusion. Changes in the volume of the intracellular compartment will clearly affect these determinations so that correction of such changes by the application of the osmotic correction factor becomes a necessity. The tests of the validity of the correction procedure performed in this present section are therefore of some importance.

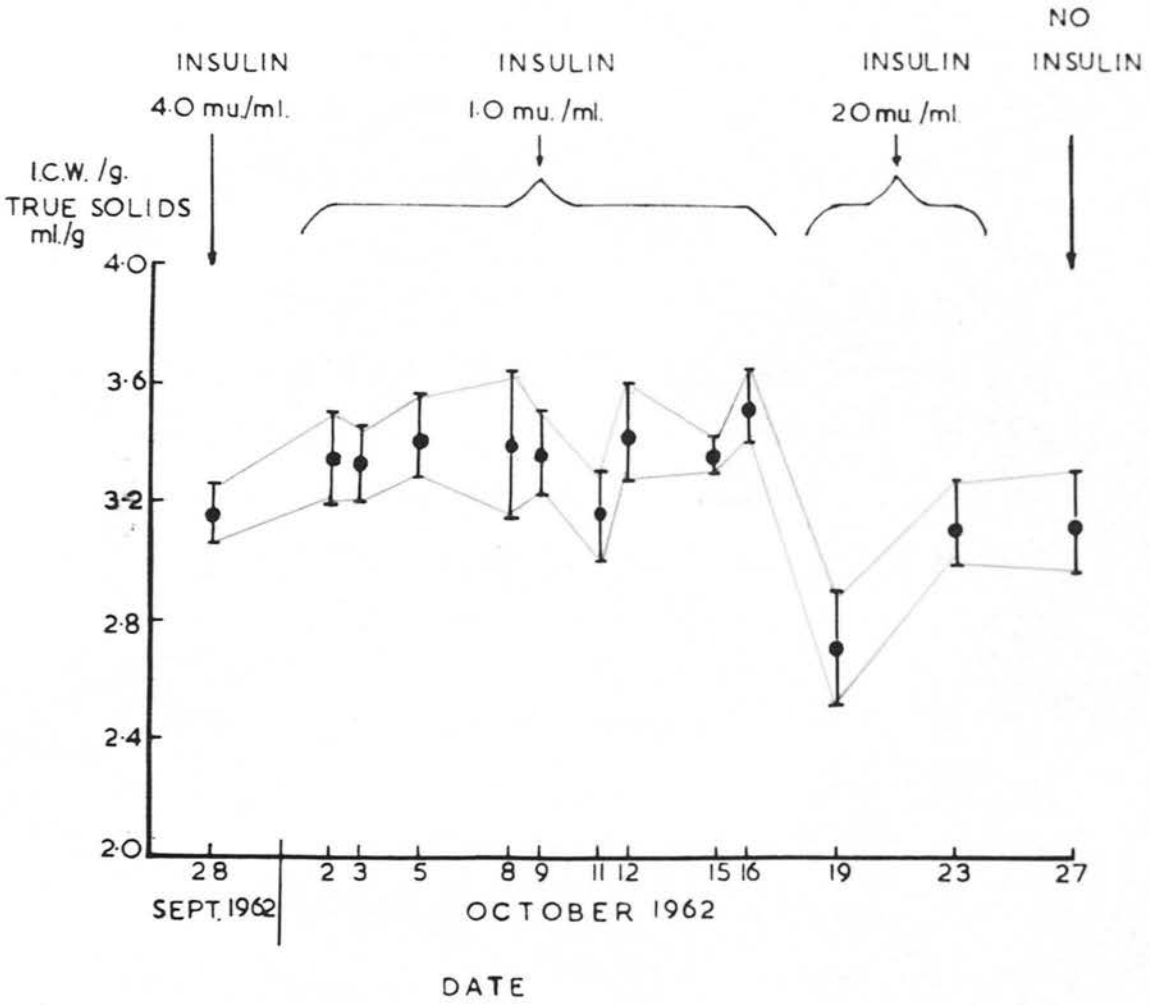
However, whilst variations in the ICW/g. true solids values due to the osmotic effects can be quantitatively corrected, the overall variability of the values was not altered by the correction. The coefficient of variation of the corrected and uncorrected ICW/g. true solids in the group of 48 hearts studied were found to be similar (Tables 18 and 24). Consequently it was not possible to establish a narrow range of ICW/g. true solids values such that hearts giving results outside that range could be considered unacceptable for perfusion results.

Insulin and cell water

The extent to which variability inherent in the animals can influence the results is well illustrated by the data concerning insulin action and the cell water. On considering the data obtained for ICW/g. true solids when the insulin and non-insulin experiments were performed at different times of the year it seemed probable that insulin significantly increased the cell water of the perfused heart (see Table 23). However, when a direct experiment was performed to test this possibility insulin and non-insulin perfusates

FIG. 26.

VARIATION IN INTRACELLULAR WATER



The points are the mean of 6 osmotically corrected intracellular water values per gram true heart solids. The lines between the points show the limits outside which the probability of a value occuring by chance is $p = 0.1$.

being used on the same days, no effect of insulin on the cell water was evident (see Table 26). On exploring the variations in the osmotically corrected ICW/g. true solids over a period of a month the pattern shown in Fig. 26 emerges. The day to day variations in osmotically corrected ICW/g. true solids have been shown to be statistically significant, and it seems to have been entirely fortuitous that a series of high values occurred at a time when most of the experiments involving insulin were performed.

These experiments on the variations in cell water per unit true heart solids points to the necessity in the experiments on sugar-uptake in the later sections of estimating always the individual intracellular water values of hearts and to the undesirability of using a pre-determined mean value for this calculation.

Electrolyte Studies

Mention should be made of the data on the distribution of electrolytes in the heart, although this is ancillary to the main problem. Ever since the work of Fenn, Cobb and Marsh (1934) and of Fenn (1936) it has been recognised that whilst

the *in vivo* chloride space of muscle corresponds to the interstitial space observed histologically (Fenn, 1936) the distribution of the ion in isolated muscles immersed in Ringer solution increases to well above this value. That chloride penetrates the cells of isolated muscle was confirmed by Boyle, Conway, Kane and O'Reilly (1941). The excess of chloride in the isolated muscle immersed in Ringer solution compared with that found in the freshly excised muscle has been explained in various ways. Eggleton, Eggleton and Hamilton (1937) attributed the difference to the adsorption of chloride ions on to the muscle fibres in the isolated muscle, and Fenn (1936) considered that both increase in the interstitial space at the expense of some muscle fibres and also changes in the permeability properties occurring with the death of fibres, contribute to the difference.

Boyle & Conway(1941) suggested that the *in vitro* preparation has a greater tolerance to potassium ion loss, so that it is possible to observe a greater entry of chloride into the cells in response to a greater loss of potassium ions in

this preparation. Whatever the reason, there is some difference in permeability properties between the two preparations which permits chloride to penetrate the cells in the one case. There is good agreement between Zachariah's (1961) estimate of the intracellular chloride concentration in the perfused rat heart, expressed as a percentage of the extracellular concentration (23%) and the result obtained here (25%).

The distribution of the potassium ion provides more interesting results. As noted by Zachariah, there is no correlation between this and the chloride ion distribution. The intracellular concentrations are well grouped about the man of 140 m.Eq./l. (Table 26), and this is further evidence that the hearts are in good condition, for there is a more or less steady loss of potassium during the life of the perfused heart (Zachariah, 1960) and values of roughly 120 m.Eq./l. are associated with impending failure. The relationship between the cell potassium concentration and the contractile properties of heart muscle has been reviewed by Haydu and Leonard (1959). The values of Table 26 are in good agreement with those of Creese (1954) for the cells of rat

diaphragm, and of Robertson and Peyser (1951) for cat ventricular muscle.

Insulin and potassium levels

It is well known that insulin affects the potassium levels of muscle (Leupin and Verzar, 1950; Kamminga, Willebrands, Groen and Blickman, 1950). Zierler (1957) has shown that although it increases the intracellular potassium concentration, this increase is insufficient to account for the alteration he observed in the resting membrane potential of the muscle. Creese and Northover (1961) also observed an insulin-stimulated potassium increase in the cells of the isolated rat diaphragm, and they suggested that more potassium ions actually become bound to the membrane. In their experiments, a decrease in diaphragm intracellular sodium concentration accompanied the potassium increase. In the present work, the cardiac cell potassium level was increased from 140 m.Eq./l. to 154 m.Eq./l. by 4.0 mu. insulin/ml. perfusate (Table 26).

This effect of insulin was obtained in the absence of glucose, and in the absence of non-metabolised pentoses, so that the effect is

independent of an effect on glucose metabolism or sugar transport.

Permeability changes in the perfused heart

When the permeability properties of the rat heart are investigated by perfusion in the apparatus including a hardened paper filter, a curve is produced (Fig. 25, curve C) which is similar to that found by Zachariah (1961) who used a sintered glass filter and who included serum protein in the perfusate (curve B). When the sintered glass filter was used in the absence of serum protein, curve A was produced (Zachariah, 1961).

With any of the sets of conditions of the perfusion experiment, the permeability of the freshly excised heart is high, it falls over the first 30 min. of perfusion, and subsequently increases at a time which is dependent upon the perfusion technique. Curves B and C each reveal a period of stable permeability, amounting to some 50 min. in B and 75 min. in C, between the two periods of changing permeability. The fall in permeability over the first 30 min. of perfusion has been shown to be due to the loss of

endogenous insulin activity from the preparation (Zachariah, 1961) so that during the subsequent stable period, the heart can be assumed to be free from such activity. This stable permeability period of curve C can therefore be used to investigate the kinetics of sugar uptake in the absence of ox-serum.

In all the experiments to be described on the kinetics of permeation, hearts have been perfused for at least 30 min. - the preperfusion period - before transferring to a medium containing the sugar to make the sugar-uptake measurement. This preperfusion period is necessary not only because of these considerations of the permeability properties, but also to allow for equilibration with the heart of the inulin and in some cases also of the insulin present in the perfusate (see p. 148).

It is interesting to note that the rate of change in permeability properties of the perfused heart over the first 30 min. is of the same magnitude whether the apparatus includes a hardened or a sintered glass filter (Fig. 25). It is therefore unlikely that the phenomenon of insulin-binding by the glass surface plays any significant part in the loss of hormone from the system.

Various reports in the literature (see below) indicate that insulin can be lost from a system in this fashion, and the possibility is particularly relevant here where the change in the filtration device results in a considerable change in the area of glass surface in contact with the perfusate. Cunningham (1962) reported that the stimulation of glucose consumption by rat diaphragm when insulin was added was enhanced by gelatin, and that the gelatin itself had no effect in the absence of the hormone. The effect was assumed to be due to a reduction in the adsorption of insulin by the glassware when gelatin was present. Other reports indicate that α-casein and bovine plasma albumin can prevent insulin adsorption by glass (Narahara and Williams, 1958). Although qualitatively this insulin adsorption phenomenon will almost certainly occur in our experiments, it is unlikely that it causes any significant loss of insulin from our system. The reasons for this conclusion are as follows. Cunningham used a total volume of incubation medium for the rat diaphragms of 1 ml. contained in a 10 ml. Erlenmeyer flask. It can be calculated that the surface area of the glassware in contact

with the medium is of the order of 8 sq. cm. when the flask is shaken during the experiment. If the insulin concentration is the highest used by Cunningham, 1.0 mU./ml. then there will be 1.0 mU./8 sq. cm. of surface. Cunningham's results indicate that under these conditions an 80% loss of the insulin activity is caused. It can be calculated from the paper of Wright (1957) that when the ratio is 1.0 mU. insulin/6 sq. cm. of glass surface, the loss in insulin activity, again measured in terms of the glucose uptake of the rat diaphragm, is approximately 30% when estimated using the calibration graph given by Cunningham. In the present work, if the perfusate insulin concentration is 1.0 mU./ml. then there will be approximately 1.0 mU. insulin/1 sq. cm. of glass surface, since the total perfusate volume is usually approximately 70 ml., and the total glass surface is approximately 80 sq. c.m. The loss of insulin activity by adsorption of insulin should therefore be very small in view of the data of Cunningham and of Wright. It will be appreciated that it is difficult to compare the results of these workers. It is more difficult in the light of their results to make assumptions

concerning insulin adsorption in the perfusion system used in the present work. Conclusions can be drawn, however, by comparing the results obtained here with those of Zachariah (1961) which are given in Fig. 25. If insulin adsorption were occurring to any significant extent in these perfusion experiments, then the fall in the permeability properties of the heart should be greater, or at least more rapid, when the sintered glass filter is included in the apparatus (curve A) for the total area of glass surface when this is present is some 20,000 sq. cm., that is 250 times greater than when a hardened paper filter is used (curve C). It will be seen that this is not the case. From these considerations it seems unlikely that a significant amount of insulin activity is lost from the perfusate by adsorption in the present experiments. It is possible, nevertheless, that adsorption of materials necessary for the normal functioning of the heart does occur during perfusion experiments, and this could contribute to the early failure of hearts under certain conditions (Fig. 25 curve A). It is possible, that it is just such a process

which Zachariah was able to offset by the inclusion of dialysed ox-serum in his perfusate, for this could prevent the adsorption of certain materials by the glass apparatus (Fig. 25 curve B).

Cardiotonic Factors

Another explanation of the beneficial effect of serum protein on the performance of the heart could be that the serum protein preparation replenishes materials lost from the heart during perfusion, and there is evidence supporting this possibility. Ringer (1885) demonstrated that the inclusion of blood in a saline medium perfusing the isolated frog heart had a beneficial effect upon the heart. Later, in 1913, Clark confirmed Ringer's finding, and further showed that the inclusion in the saline perfusate of a lipid extract of blood would also permit the frog heart to function efficiently for a longer period than if it was perfused with saline medium alone. Clark noted too that the onset of the hypodynamic changes in the heart could be delayed by cutting down the volume of the medium perfusing the organ. This led to the suggestion that the process of perfusion washed-out endogenous materials having a beneficial effect upon the heart.

Haydu and his colleagues have made extensive studies of cardiotonic materials and have described two systems in good detail, (Haydu, Weiss and Titus, 1957; Haydu and Leonard, 1958 and 1961). They assign a powerful cardiotonic action to β-palmitoyl lysolecithin, a normal constituent of adrenal medulla and heart muscle, but not of skeletal muscle. A more complex system described by these workers consists of 3 globulin components present in the plasma of patients suffering from hypertension. The biological activity of the system seems to depend upon the carriage of calcium ions to the heart contractile mechanism.

These considerations of cardiotonic factors make it understandable why preparations of perfused hearts, (Clark, 1913; Haydu et al. 1957) usually become hypodynamic in a comparatively short time, whereas unperfused preparations, as for instance the cat papillary muscle of White and Salter (1946) can maintain normal contractility for many hours. It is true the lower oxygen consumption found for the unperfused preparation may reflect its poorer functional state and this could mask signs of further deterioration (Zachariah, 1960) but the effect of continuous washing of the

perfused tissues is more likely to be the major cause of their earlier failure.

In the present studies, it is essential to maintain a large volume of medium perfusing the heart, so that the sugar concentration outside the heart cells can be taken as constant. This obviates the difficulty of evaluating sugar-uptake results obtained in the presence of changing perfusate sugar concentrations. In spite of the large perfusate volume however, if the sintered glass filter is replaced in the perfusion apparatus by a hardened paper filter, any loss of cardiotonic materials from the heart does not seem to significantly affect the performance of the heart over at least a 2 hr. perfusion period. With the perfusion technique finally adopted in the present work, therefore, the introduction of unknown factors due to the presence of ox-serum in the perfusate has been avoided.

CHAPTER V

Summary

1. A study has been made of the suitability of inulin as an extracellular space marker in the perfused heart.

2. As judged by its speed of equilibration with the interstitial water, by the lack of effect of other perfusate components upon its volume of distribution and by the agreement between the inulin space and the extracellular spaces given by other methods, inulin is an adequate extracellular marker in the perfused heart.

3. The volume of the intracellular water of the heart varies in a fashion which is predictable on the basis of the concentrations of osmotically active materials contained in the medium perfusing the heart. This finding supports the validity of the techniques involved in the intracellular water measurement.

4. The volume of distribution of the chloride ion and the intracellular concentration of potassium in the heart are both in agreement with the results obtained by Zachariah for heart preparations showing normal contractility.

5. When the sintered glass filter is replaced in the perfusion apparatus by a paper filter, hearts will survive by perfusion for over 2 hr. in spite of the omission of plasma protein from the perfusate.

6. Over a long period of perfusion the permeability properties of hearts tend to change when perfused with a modified Krebs bicarbonate medium containing nutrient but no plasma protein. However, after 30 min. of perfusion, when endogenous insulin activity has been lost, a period of stable permeability extending over some 75 min. occurs and this period is considered suitable for sugar transport studies.

SECTION IV

THE NATURE OF THE SUGAR TRANSPORT PROCESS

CHAPTER I

Introduction

The object of this section of the thesis is to ascertain whether sugar uptake by the cells of the perfused heart conforms to the kinetics of simple diffusion or to carrier transport kinetics, for although the main body of evidence indicates that sugar transport is mediated by a carrier mechanism, it is felt that this point should be demonstrated unequivocally. This is best achieved by evaluating sugar uptake for particular intervals of time at a series of perfusate sugar concentrations. It can then be determined whether the uptake is proportional to the perfusate sugar concentration as demanded by diffusion kinetics, or whether the uptake is inversely proportional to the perfusate sugar concentration as would be expected on the basis of a carrier mechanism. Furthermore, if the latter is found to be the case, then the conformity of the sugar uptake results to the permeation equation (Section I, p. 31) should be tested at all the perfusate sugar concentrations studied, for this would further test the validity of the carrier trans port model.

There is a considerable amount of work reported in the literature claiming that sugar transport conforms to simple diffusion kinetics, (Kipnis and Cori, 1957; Resnick and Hechter, 1957; Norman, Menozzi, Reid, Lester and Hechter, 1961), but all of this work is based on the assumption that a large proportion of the cell water of tissues is not accessible to sugars. In addition, the work of Morgan, Cadenas, Regen and Park (1961) which was submitted in support of the carrier hypothesis is based on the assumption that only 75% of the cell water of the perfused heart is available to the penetrating sugar. By contrast, the sugar uptake measurements of Fisher and Zachariah, (1961) which provided the stimulus to the present work, were based on the premise that 100% of the cell water is available to the penetrating sugar.

It will be seen later (p. 189) that these assumptions as to the availability of the cell water can profoundly influence the compatibility of sugar uptake results to the various kinetic schemes proposed for the sugar transport process. Therefore, before performing the experiments

outlined in the early part of this introduction, it is clearly necessary to establish by direct experiment the maximum ~~penetration~~ percentage of the cell water of the perfused heart which can be penetrated by sugar.

CHAPTER II

Methods

The animals used and the composition of the perfusion medium are as stated in the previous section (p. 90). The perfusion technique has also been described earlier, Section III, p. 91).

Hearts were preperfused for a period of 30 min. to free them of endogenous insulin activity, and then transferred to media containing sugar in the concentrations indicated for different perfusion times*. When insulin was used it was included in both preperfusion and perfusion media. The preparation of heart extracts and the determinations of pentose sugars and the extracellular marker inulin were performed as indicated in Sections II and III. The extent of penetration of the cell water by the sugar is expressed as percentage penetration, the method of calculation being given in Section III.

*The preperfusion medium was always the modified Krebs bicarbonate medium given in Table 9 of the last section, and it always contained 4mM sodium pyruvate and 2% inulin. This medium will be referred to as the 'basic medium'.

CHAPTER III

Results

Maximal Penetration of Cell Water by Sugars

The volume of intracellular water available to the penetrating sugar in the absence of insulin was estimated by transferring three preperfused hearts to a medium containing approximately 4mM xylose for 75 min. The time of perfusion was estimated by using the sugar transport equation in the form (see eq. (1) Section I, p.1)

$$\frac{(K + x)^2}{KV} \ln \frac{1}{1-f} - x\frac{(K + x)}{KV} f = t$$

and by substituting in this equation values of K and V estimated from results to be presented later. The mean percentage penetrations of the cell water is given in Table 28. It is well in excess of the values claimed by other workers to be the maximal possible penetration (see Discussion, p.185).

Maximal Penetration of Cell Water by Sugars in the Presence of Insulin

Hearts were preperfused in the presence of insulin in concentration approximately 4.0 mu./ml. and then transferred to a similar medium containing 15mM L-arabinose and perfused for 35 min.

TABLE 28

Volumes of Distribution of Sugars in Cell Water

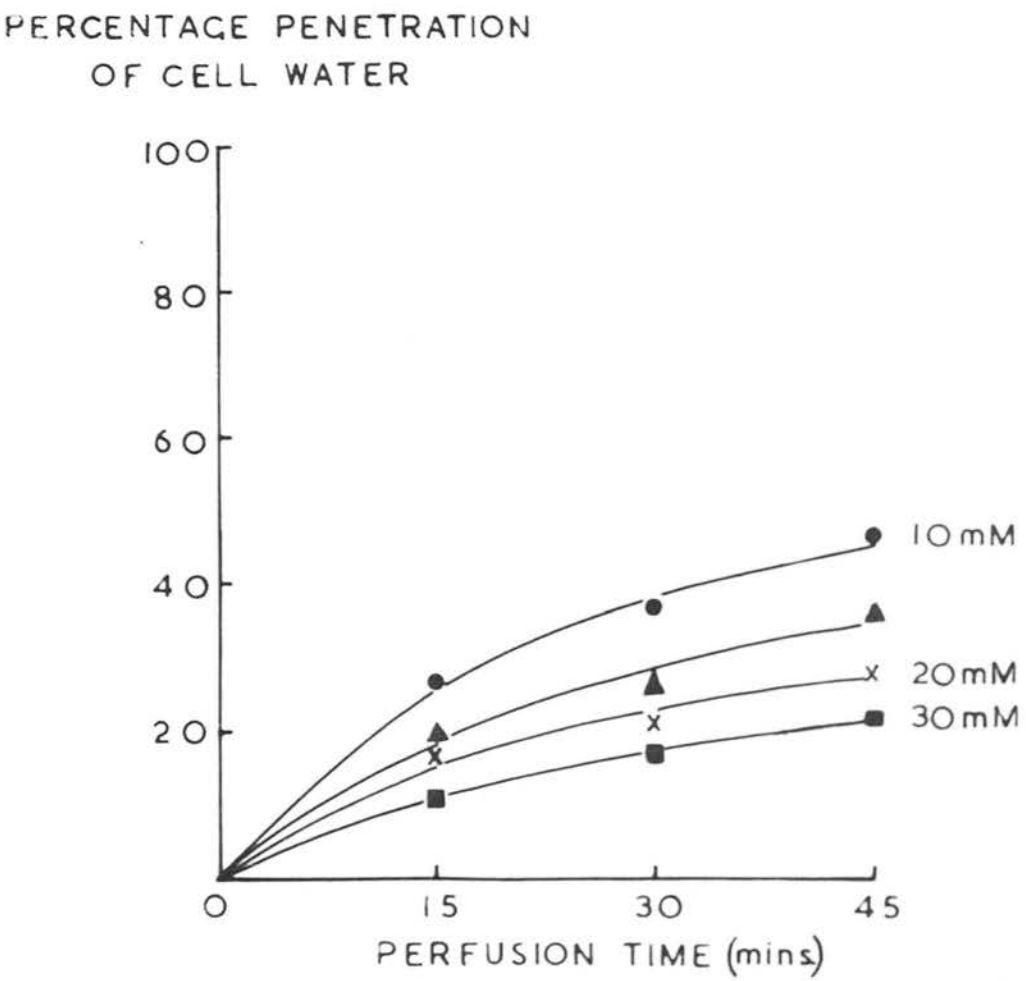
Sugar	Concentration (mM)	Insulin (mU./ml.)	Perfusion Time (min.)	% Penetration ± SEM
D-Xylose	4	nil	75	95.8 ± 2.8 (3)
L-Arabinose	15	4.0	35	104.0 ± 3.9 (3)

The number of observations is given in parenthesis

Hearts were preperfused for 30 min. on a medium containing 4mM sodium pyruvate and 2% inulin, and then transferred to a second medium containing xylose or arabinose in the concentrations shown. The hearts were perfused on the second medium for 75 min. in the case of xylose, and 35 min. in the case of arabinose. Insulin, when used, was present in both pre-perfusion and perfusion media.

FIG. 27.

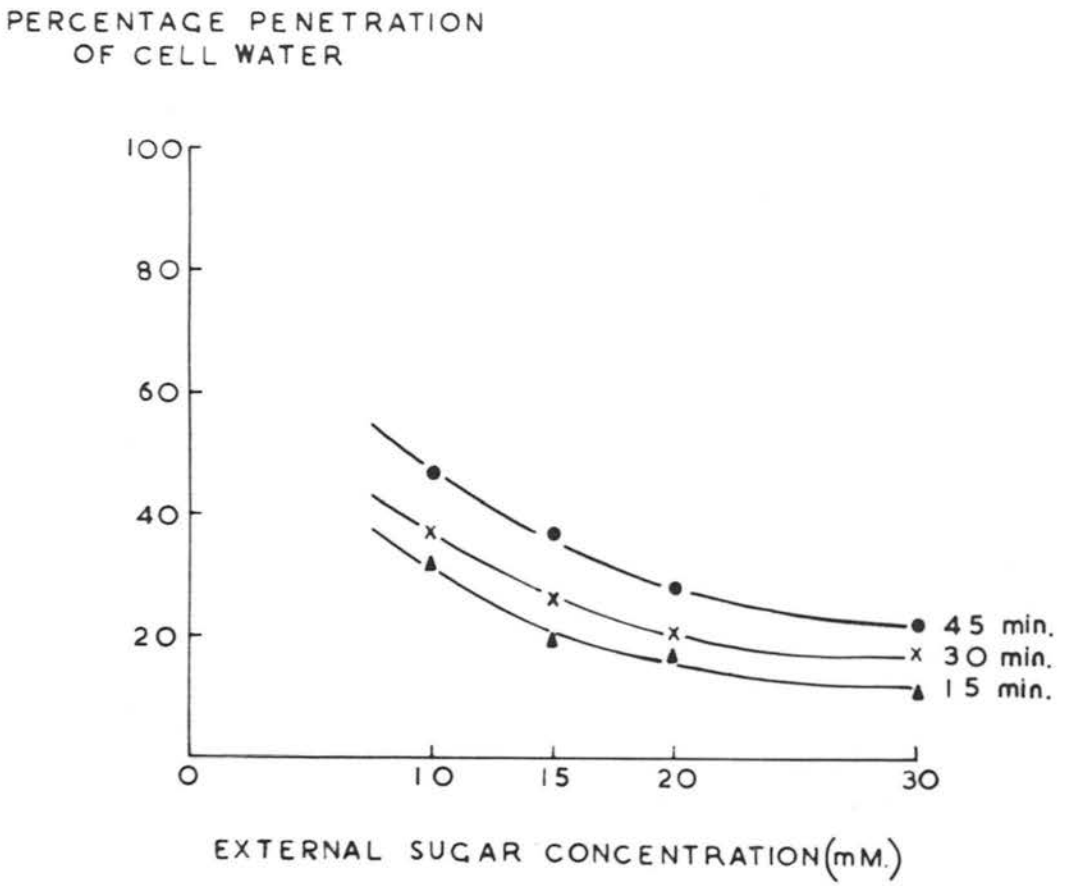
TIME COURSE OF L-ARABINOSE UPTAKE FOR DIFFERENT EXTERNAL SUGAR CONCS.



The points are the mean observed values as given in Table 29. The curves through the points are drawn by eye.

FIG. 28.

EFFECT OF CHANGES IN EXTERNAL SUGAR CONCENTRATION ON UPTAKE OF L ARABINOSE OVER DIFFERENT TIME INTERVALS BY THE PERFUSED HEART.



The points are the mean observed values given in Table 29. Standard errors have been omitted for the sake of clarity. The lines through the points have been drawn by eye.

This perfusion time was calculated using values of K and V to be presented later, as indicated above. The mean penetration of cell water by the sugar is given in Table 28. All the cell water can be assumed to be accessible to sugar. The excess of the mean penetration above 100% indicates the variability inherent in the experiment.

Effect of Change in the Perfusate Sugar Concentration on the Penetration of Sugar into the Heart cells

The effect of change in the perfusate sugar concentration on the percentage penetration of cell water by the sugar was investigated as follows. Preperfused hearts were transferred to media containing L-arabinose for 15, 30 or 45 min. The concentrations of arabinose in these media were 10, 15, 20 and 30mM. The percentage penetrations of cell water by arabinose are given in Table 29.

The results of a similar experiment employing D-xylose at the four concentrations are given in Table 30 for the 45 min. perfusion time only. The arabinose results are plotted in Figs. 27 and 28 and the standard errors of the mean penetrations

TABLE 29

Arabinose Uptakes by Hearts Perfused With Different Sugar Concentrations

Perfusion Time(min.)	Perfusate Sugar Concentration			
	10 mM.	15 mM.	20 mM.	30 mM.
15	17.7	34.4	11.0	11.7
	13.9	9.3	17.5	23.2
	53.9	17.7	23.6	4.6
	25.5	12.0	26.4	7.6
	24.3	23.4	6.5	6.9
Mean	27.1 (5)	19.4 (5)	17.0 (5)	10.8 (5)
SEM	±7.0	±4.5	±2.9	±3.3
30	51.5	25.0	27.1	22.2
	35.7	36.4	26.7	19.7
	50.8	18.2	25.1	23.9
	27.9	17.4	14.5	4.4
	18.7	34.5	11.0	
Mean	36.9 (5)	26.3 (5)	20.9 (5)	17.5 (4)
SEM	±6.4	±4.0	±3.4	±3.5
45	39.3	25.9	20.9	26.0
	56.1	55.7	44.1	35.5
	39.6	28.9	20.4	19.6
	40.9	46.6	28.8	17.6
	56.9	26.1	26.3	12.6
Mean	46.6 (5)	36.6 (5)	28.1 (5)	22.3 (5)
SEM	±4.1	±6.1	±4.3	±4.0

The figures represent the percentage penetrations of cell water. Hearts were preperfused with the basic medium for 30 min. before transference to the sugar-containing media for the times shown.

TABLE 30

Uptake of D-Xylose by Rat Hearts
Perfused for a 45 min. Period
With Different Sugar Concentrations

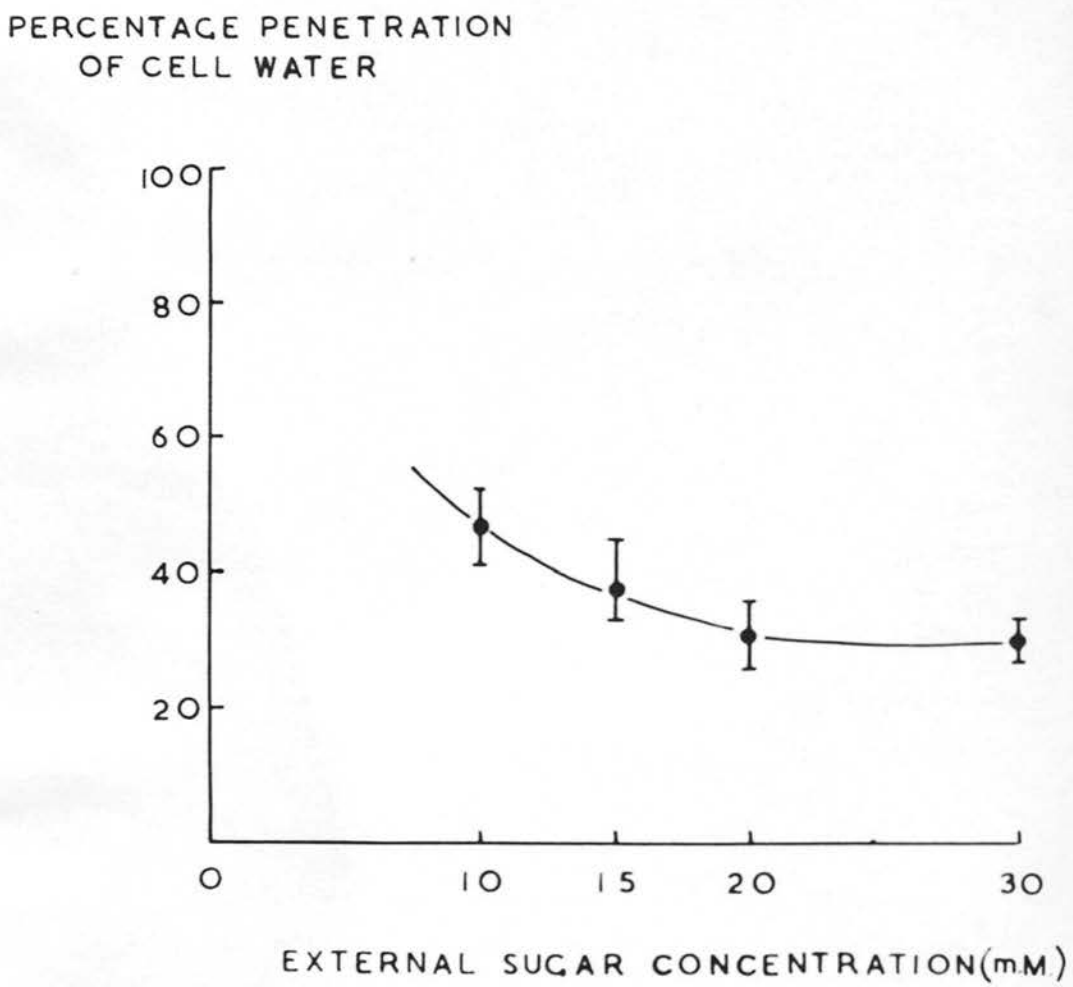
	Perfusate Sugar Concentration			
	10 mM.	15 mM.	20 mM.	30 mM.
	63.0	31.4	24.3	30.2
	44.9	29.1	35.3	23.6
	45.1	55.3	42.4	29.0
	34.3	40.5	21.7	38.6
Mean	46.8	39.1	30.9	30.3
SEM	±6.0	±5.9	±4.8	±3.1

The figures represent the percentage penetrations of cell water.

Hearts were preperfused for 30 min. on the basic medium before being transferred to the xylose-containing medium for the times shown.

FIG. 29.

UPTAKE OF D-XYLOSE OVER A 15 MINUTE PERIOD AT DIFFERENT EXTERNAL SUGAR CONCENTRATIONS



The points are the mean observed values ± SEM given in Table 30. The curve through the points was drawn by eye.

have been omitted for the sake of clarity. They are a little less than those shown in Fig. 29 which illustrates the xylose results. The curves through the points have been drawn by eye.

It will be seen from the arabinose results of Fig. 27 that the entry of sugar into the heart cells increases with the perfusion time, but there is a fall off in the rate of transport as the perfusion time increases, and this is most pronounced over the first 15 min.

The same form of sugar penetration curve is found at all four sugar concentrations studied. When the perfusate sugar concentration is decreased so a greater penetration of the cell water by the sugar is achieved. This is best illustrated by Fig. 28 for arabinose and Fig. 29 for xylose. There is therefore an inverse relationship between the rate of sugar uptake by the cardiac muscle cells and the concentration of sugar in the perfusate.

A further test of the conformity of these results to the carrier transport model as it is investigated in the present work will be made in the final section.

CHAPTER IV

Discussion

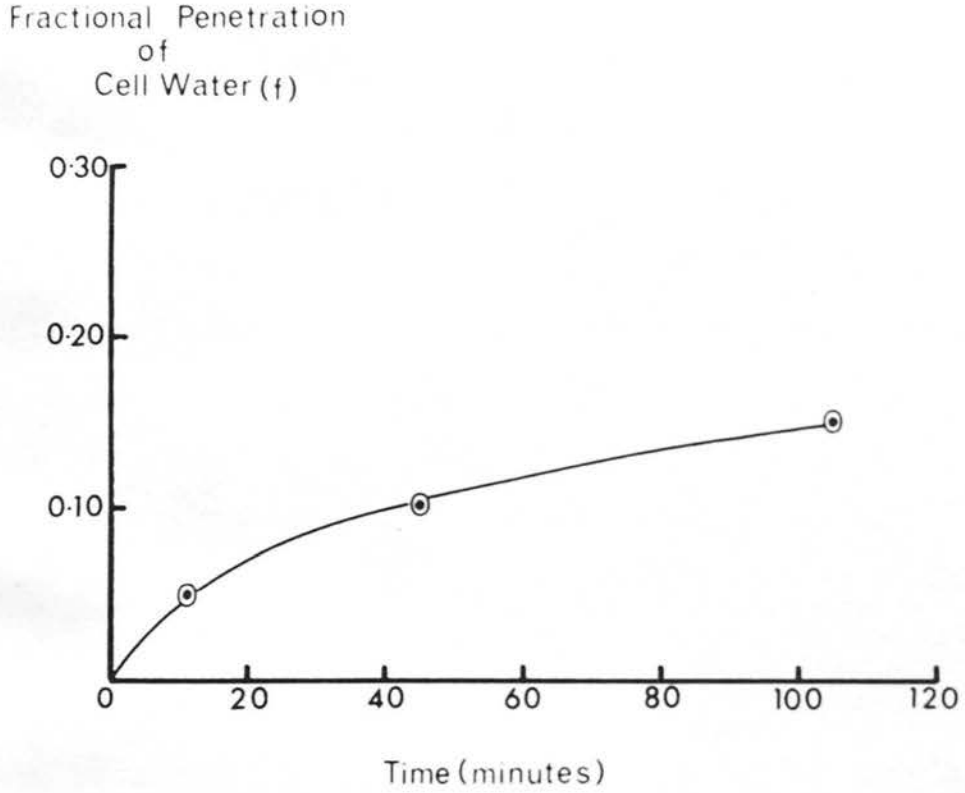
The results presented here serve to show not only that the entire volume of intracellular water is accessible to the sugar being transported into the cell from an external medium, but also why some workers have assumed this not to be the case. This latter possibility stems largely from studies on the volume of distribution of a sugar and the effect of insulin on this volume. For example, Helmreich and Cori (1957) found not only that the distribution of pentoses in rat gastrocnemius muscle was increased by insulin, but also the utilisation of the sugars. They suggested that insulin might remove certain intracellular barriers. This would permit a greater penetration of sugar through the cell water, and at the same time give access of substrate to a larger amount of enzyme. Again, in studies using the isolated rat diaphragm Randle and Smith (1958) suggested that an intracellular barrier restricts xylose distribution to a small fraction, of the order of 20% of the cell water, and that insulin permits the pentose to penetrate to some 40 - 80%. And so the concept

of different aqueous compartments within the cell has been evolved, and this could tend to direct attention away from the cell membrane as a locus of insulin action. No one doubts the existence of different intracellular compartments within the cell, the intranuclear and intramitochondrial water being apparently distinct from the cytoplasm but it is unlikely that the volume occupied in the cell by the nucleus and mitochondria is sufficiently large to account for the observations of, for instance, Randle and Smith. It has been possible, using electron micrographs of the perfused rat heart described in the final section of this thesis, to estimate the approximate volume of the cell taken up by these structures. By tracing the areas of the cell and its inclusions from the micrographs and placing the tracings over graph paper, it has been found that the cell inclusions occupy approximately 20% of the total area of the micrographs. The volume occupied by the inclusions is likely to be of this same order. In fixed preparations such as those used to obtain the electron micrographs, there is frequently some 10% shrinkage of the tissue, but this should

not alter the proportions of the mitochondrial volume to the cell volume. However, in unfavourable conditions, it is known that extensive mitochondrial swelling occurs (Fonnescu and Davies, 1956). Consequently it is possible that our estimate of the mitochondrial volume compared to that of the whole cell is too large by a factor of two or three, so that the true proportion of the total cell volume might be only 7%. Another factor which must be considered is the proportion of the mitochondrial volume which is, in fact, water. It can be estimated from the data of Fonnescu and Davies (1956) that the water content of liver mitochondria is of the order of 60% and as such is considerably less than the water content of the cell as a whole. In this case, while the percentage of the total cell volume occupied by mitochondria in the heart cell might be 7% - 20% depending on whether or not swelling occurred, the intramitochondrial water would occupy only 4 - 12% of the total cell water. Thus, from 88% to 96% of the cell water is likely to be accessible to sugars assuming that the mitochondria and other particles are not penetrable;

FIG. 30.

THEORETICAL CURVE FOR PENETRATION OF CELL WATER BY SUGAR AT DIFFERENT TIMES ON THE BASIS OF THE CARRIER HYPOTHESIS



The values were calculated on the basis of the equation

$$\frac{(K + x)^2}{KV} \ln \frac{1}{1-f} - \frac{x(K + x)}{KV} f = t$$

x = 30mM; K = 0.05 mM; V = 2.2mM/min.

f values were 0.05, 0.10, 0.15 and 0.20, but the last point has been omitted from the curve for convenience.

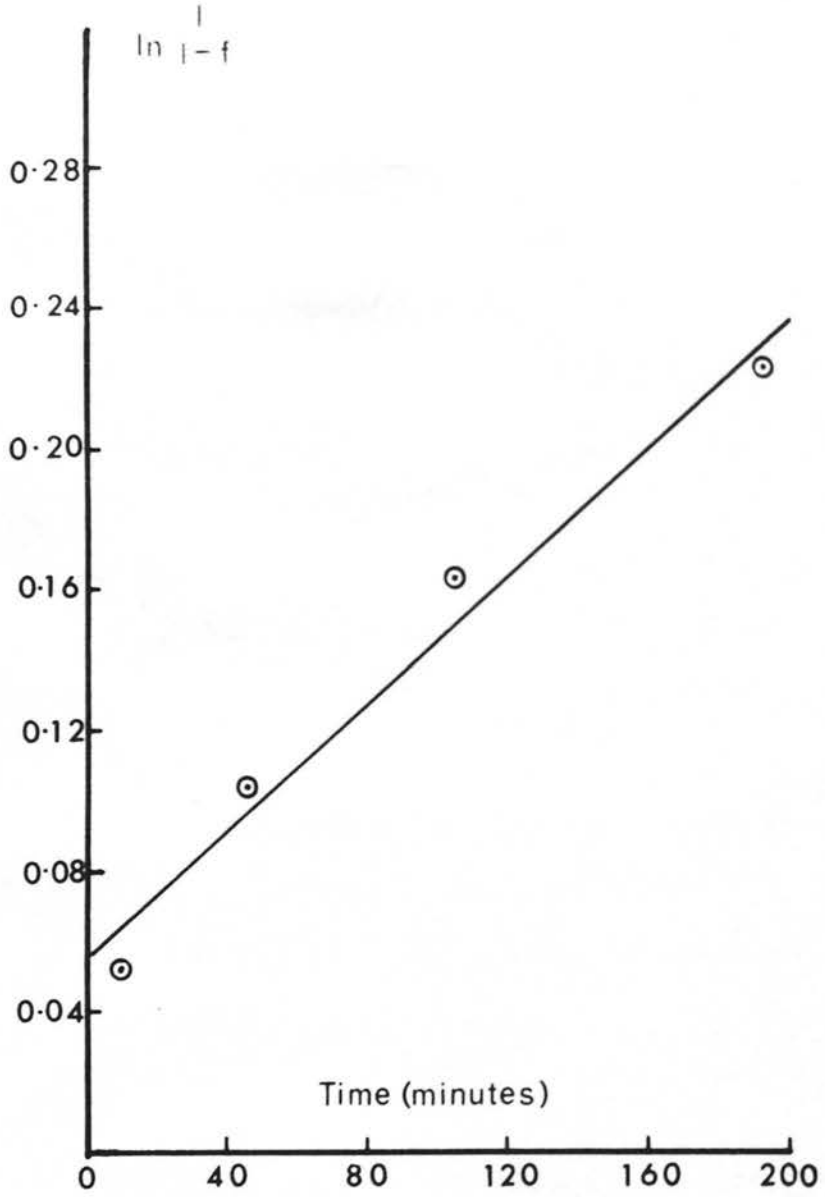
and there is evidence that simple sugars can, in fact, readily cross the membranes of these cell inclusions (Werkheiser and Bartley, 1957). Thus, it is difficult, on the basis of these considerations, to justify the assumptions of Kipnis and Cori, (1957), Randle and Smith (1958) and Morgan, Cadenas, Regen and Park, (1961) that from 20% to 80% of the cell water is not accessible to the sugar entering the cell. In spite of these reasons for excluding the notion that there are large compartments within the cell whose volumes are inaccessible to sugars, consideration of Fig. 30 indicates how one might assume that sugar penetration could not attain 100%. To obtain this figure, values of $\ln \frac{1}{1-f}$ have been computed for arbitrarily chosen values of f and then the values have been substituted in the equation given below, together with values of K and V which will be justified in Section V, so that values of t are determined.

$$\frac{(K + x)^2}{KV} \ln \frac{1}{1-f} - x\frac{(K + x)}{KV} f = t$$

Thus Fig. 30 gives the theoretical relationship between f and t on the basis of the carrier trans-

FIG. 31.

FIT OF RESULTS CALCULATED USING THE CARRIER EQUATION TO DIFFUSION KINETICS

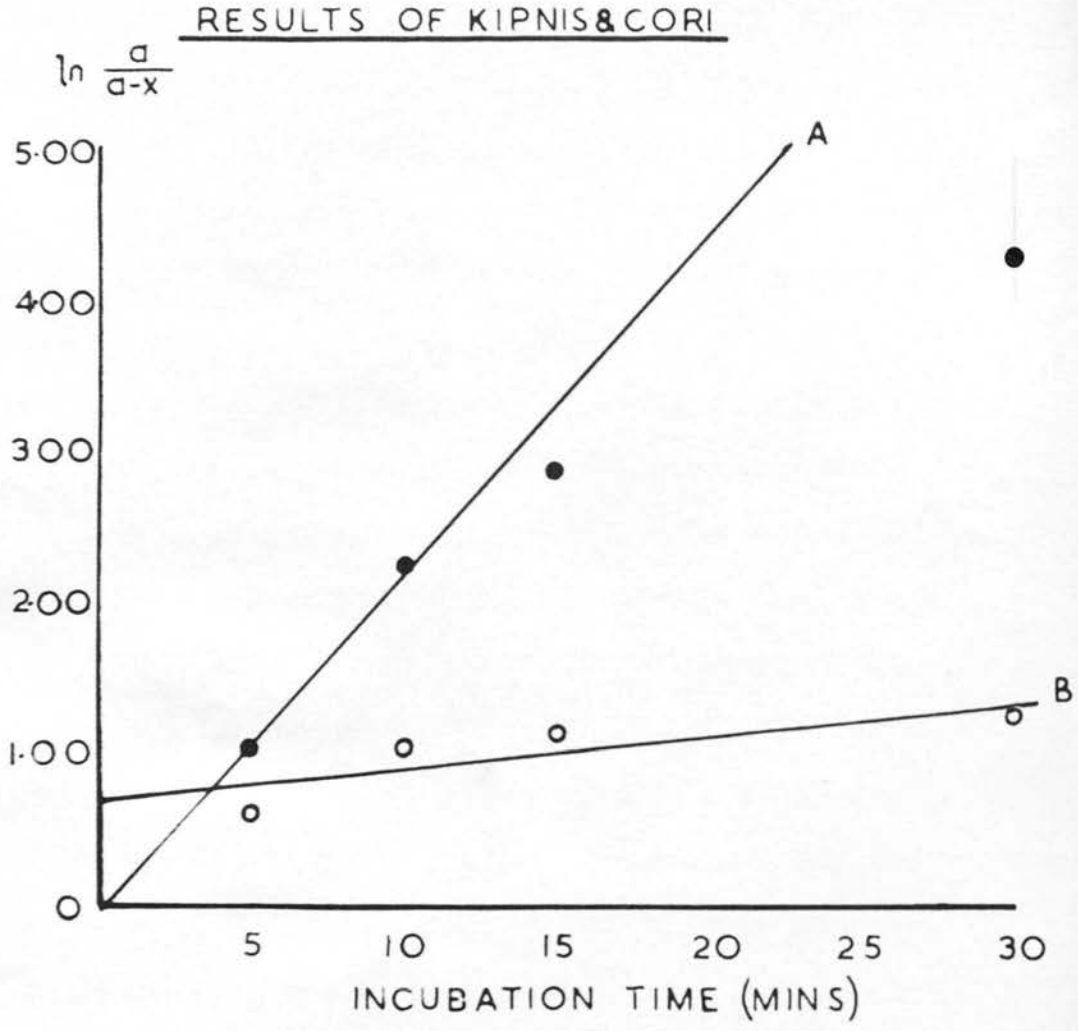


The values of $\ln \frac{1}{1-f}$ correspond to the values of f given in Fig. 30 at the particular times

port model. It will be seen that the form of the curve is similar to the curves given by the results of this section in Fig. 27. The rate of sugar transport falls off so rapidly that at longer perfusion times the rate can be experimentally indistinguishable from an equilibrium and one might assume that a value of f equal to unity can never be attained. This point provides the basis for the evidence of various workers that sugar uptake conforms to diffusion kinetics. Thus, if the values of $\ln \frac{1}{1-f}$ used in the calculation of t on the basis of the carrier equation given above are plotted against these values of t, the graph shown in Fig. 31. is obtained. It will be seen that the points conform reasonably well to a straight line and this does not pass through the origin. If one assumes, as other workers have done (Kipnis and Cori, 1957) that some of the cell water is not accessible to sugar, then these results can be interpreted as being compatible with first order reaction kinetics.

For comparison with Fig. 31, the results obtained by Kipnis and Cori (1957) for the uptake of xylose at four different incubation times by

FIG. 32.



The points are calculated from the xylose-uptake data given in Table 31.

Line A has a slope of 0.22 and correspond to the rate constant calculated by Kipnis and Cori.

Line B is produced if all the cell water is assumed accessible to sugar.

the cut diaphragm preparation are given in Table 31 and illustrated by filled circles in Fig. 32. No such figure was included in the paper of Kipnis and Cori, for they simply calculated the rate constants of sugar uptake on the basis that only 60 - 70% of the cell water is available to the xylose. In Fig. 32 the line 'A' has a slope of 0.22 and corresponds to the rate constant calculated by the workers using the first order reaction equation.

$$k = \frac{1}{t} \ln \frac{a}{a-x}$$

The points (filled circles) conform to this line only over the first 10 min. of incubation of the diaphragm, and cannot be considered compatible with first order kinetics. The results have been re-interpreted on the basis that sugar can penetrate all the cell water, and the plot is also shown in Fig. 32 (open circles, line B). The points form a reasonable straight line and this does not pass through the origin. The results cannot be considered as evidence for a diffusion mechanism.

TABLE 31

Rate of Penetration and Distribution Equilibrium of D-Xylose in Cut Diaphragm Preparation

Xylose concentration 5.0 mg./ml. Insulin concentration 0.4 u/ml.

Incubation Time (min.)	5	10	15	30	60
Intracellular Pentose Concentration (mg./ml.)	2.33	3.20	3.38	3.53	3.58
$\text{Ln} \frac{a}{a - x}$	1.05	2.24	2.88	4.27	
Recalculated $\text{Ln} \frac{a}{a - x}$	0.63	1.03	1.14	1.24	1.28

First order velocity constant given as 0.22 min.^{-1}

Final volume of distribution 72%

It should be appreciated, therefore, that the results of sugar uptake by a carrier transport mechanism can be interpreted as obeying simple diffusion kinetics if a large volume of the cell water of the preparation is assumed to be inaccessible to sugars. The results of the first part of this section, (p. 180) however, show conclusively that providing a low concentration of sugar is employed in the perfusate, it is readily demonstrated that all of the cell water is available to the penetrating sugar, so that the claim of other workers that the kinetics of transport are compatible with the process of diffusion of the sugar into a restricted volume of the cell water is not valid.

The data obtained here from the sugar uptake studies at different external sugar concentrations provides more direct evidence that a carrier mechanims and not simple diffusion mediates sugar transport. If a diffusion process were involved, the rate of sugar uptake would be directly proportional to the external sugar concentrations and the percentage penetration at any given time should be independent of the concentration of sugar in the medium. In fact the percentage penetration

decreases as the perfusate sugar concentration is increased, (Fig. 28), indicating that sugar uptake fails to keep pace with increases in the perfusate sugar concentration and this occurs at all three perfusion times studied. This phenomenon is readily explicable on the basis of the carrier hypothesis (Section I p. 30). These experimental data are examined in greater detail in the next section.

It is felt, therefore, that by far the greater body of evidence favours the carrier mechanism as the means by which sugar is transported into muscle cells. In the final section, the main features of such a mechanism are investigated and the effects of insulin on sugar transport are examined in the light of the carrier hypothesis.

CHAPTER V

Summary

1. In the perfused heart experiments indicated that all of the intracellular water may be assumed to be available for penetration by sugars both in the presence and absence of insulin.

2. Over the range of concentrations studied here, for both xylose and arabinose, the percentage penetration of the cell water by sugar in any particular time is inversely related to the perfusate sugar concentration.

3. Evidence on the kinetics of sugar transport has been discussed and it is concluded that the sound evidence favours a carrier rather than a diffusion mechanism.

SECTION V

THE MECHANISM OF ACTION OF INSULIN ON SUGAR TRANSPORT

CHAPTER I

Introduction

It has been shown in Section I that the carrier model of sugar transport can be treated mathematically so that it yields a permeation equation which should permit a direct evaluation of the parameters of the transport process - K, the apparent dissociation constant of the sugar-carrier complex, and V, the rate constant of maximal transport. The object of this final section is to investigate the time-course of sugar-uptake by the cells of the perfused heart, and to examine the results in the light of the permeation equation. If the sugar uptake results conform with this permeation model, both in the presence and absence of insulin, then the changes induced by insulin in the values of K and V can be assessed and these should clarify the mechanism of action of the hormone. The results of these investigations can then be compared with those obtained by Fisher and Zachariah (1961) using the same approach, and the hypothesis of insulin action formulated from that earlier work will therefore be further tested.

In view of the advance in perfusion technique accompanying the exclusion of serum protein from the perfusate, and the refinements made to the method of estimating the parameters from the results of sugar uptake experiments, the work described here should test the hypothesis in a thorough and reliable fashion. In this connection the consequences of the sugar transport model and of the hypothesis of insulin action which are put to test in this section should be mentioned again.

(i) In the absence of insulin, the value of K for either of xylose or arabinose, the two sugars studied, should be independent of the concentration of sugar outside the heart cells in any experiment.

(ii) On the addition of sufficient insulin to produce any noticeable increase in pentose permeation, the value of K should increase markedly, and should remain at this higher value at all higher insulin concentrations.

(iii) The value of V should diminish on the addition of a submaximal concentration of insulin but should then increase as the concentration is

raised until, at some sufficiently high concentration of insulin, V should attain again the same value as that found in the absence of hormone.

It is necessary, too, in this section to re-examine the data on sugar uptake obtained in Section IV using different perfusate sugar concentrations in the absence of insulin. The compatibility of these data must be tested not only with the permeation model, but also with the particular values of K and V obtained in this final section. These earlier data were not examined for conformity to the model at the time they were obtained because the inherent variability of the perfusion technique makes results unreliable for estimating K and V unless six or more mean values of f and t can be obtained. Thus K and V could not be determined with any reliability from the values of f obtained in Section IV since only three perfusion times were studied at any one sugar concentration. The data can nevertheless be of value in this respect once K and V have been reasonably accurately determined from the data obtained in this section.

On giving consideration to the values of K and V determined in this section, and to their significance in relation to the mechanism of action of insulin, it became clear that it would be profitable to include in this section experiments of an entirely different nature to those so far described. It was felt that the significance of the changes in K and V occurring when insulin is added could be better interpreted after investigating the possible influence on sugar transport of pinocytosis under the conditions of our experiments. There are reports in the literature that the process of pinocytosis can be implicated in the permeability properties of certain cells (Chapman-Andresen and Holter, 1955; Palade, 1956). Furthermore, there is some evidence that such a process might also be implicated in the mechanism of action of insulin on cell permeability (Barrnett and Ball, 1960). It was therefore felt that the incidence of pinocytosis in the cardiac muscle cell, both in the presence and absence of insulin, should be investigated here.

CHAPTER II

Methods

The perfusion technique used in this section is slightly different from that previously adopted (see below) but the media used are as given in Section IV, that is, the plain medium and this same medium containing the pentose. When Insulin is used in an experiment, it is included in both preperfusion and perfusion media. The preparation of heart extracts and the methods of determination of the pentose sugars and of inulin have been described.

Perfusion technique

The change in the technique concerns the preperfusion time. Fisher and Zachariah (1961) used a 30 min. preperfusion period, and this period was at first considered adequate for the previous experiments in view of (i) the rate of equilibration of inulin with the interstitial water of the heart and (ii) the attainment of the period of stable permeability of the heart only after approximately 30 min. of perfusion.

However, it was felt that it might be possible to diminish the variability of sugar

penetration results by extending the preperfusion period to 40 min. so that there is little chance of interference with these results of any endogenous insulin activity (see Fig. 25 p.144) The preperfusion period used in the experiments of this section is therefore 40 min.

Experiments on pinocytosis

In the investigation of pinocytosis, hearts were preperfused for 40 min. on the plain medium with or without insulin, and then transferred to an -arabinose-containing medium with or without insulin, for 10 min. The hearts were then fixed by the injection of 5 ml. of 1% osmium tetroxide in veronal buffer pH 7.4 into the rubber tube leading to the heart cannula. After cutting the hearts from the cannula, it was allowed to stand in a vessel containing the fixative for 30 min. Portions were then dehydrated in ethanol, embedded in araldite, and sectioned on a Porter-Blum microtome using a glass knife. Sections were stained with a 1% solution of potassium permanganate prior to examination in an A.E.I. em-6 microscope. Dr. A. Muir of the Anatomy department of this University kindly undertook to section the hearts and to prepare and examine the electron micrographs.

CHAPTER III

Results

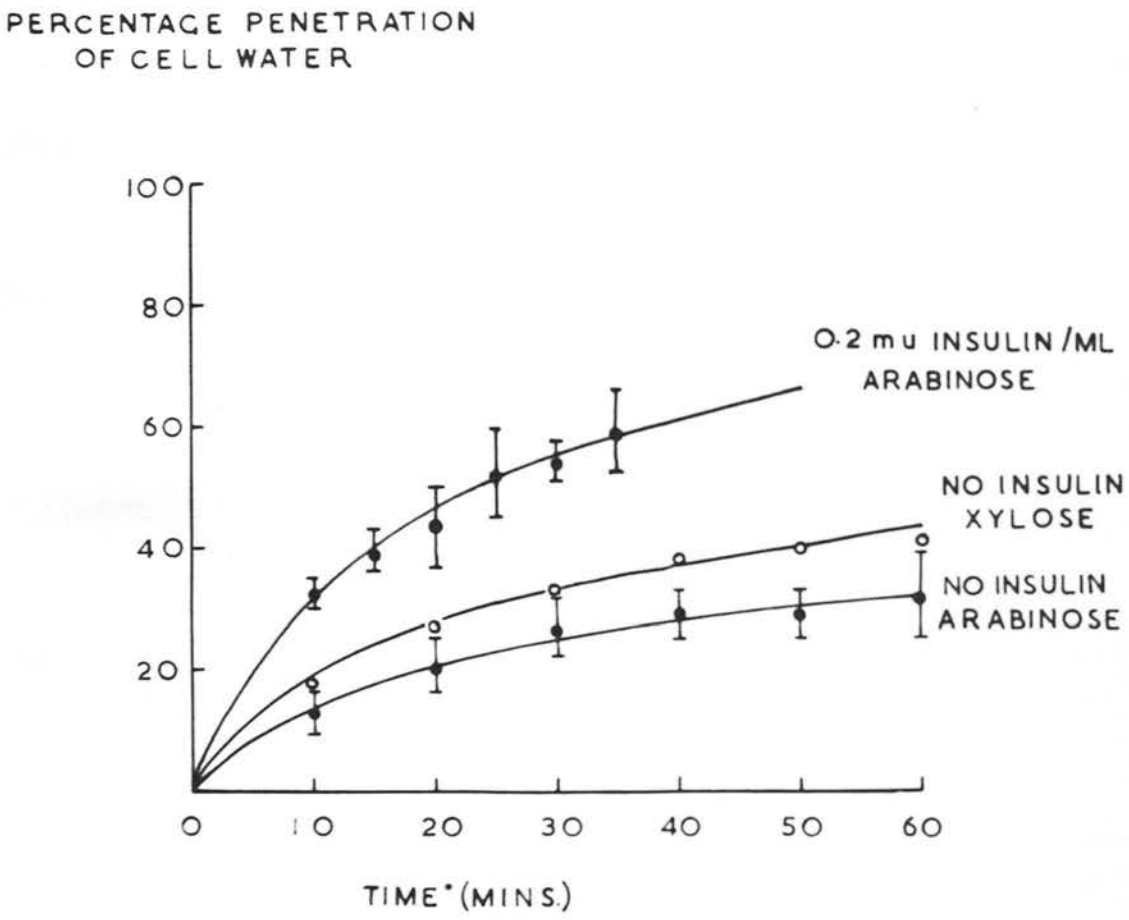
Time course of sugar-uptake

The time course of uptake of L-arabinose was studied in the absence of insulin by preperfusing hearts for 40 min. with the basic medium and then transferring them to a similar medium containing approximately 10mM L-arabinose for periods of 10, 20, 30, 40, 50 and 60 min. The percentage penetrations of the cell water by the sugar were then estimated as previously described.

Similar experiments were performed with insulin in both preperfusion and perfusion media in a final concentration of 0.2 mU/ml. The times of perfusion were different in this experiment because of the faster rate of sugar entry which was to be anticipated from the results of other workers (Fisher and Zachariah, 1961) when insulin is present. In all the experiments described all 6 perfusion times have been studied on any one day as far as possible so that any day to day variation should not influence the relationship between the fractional penetration of cell water by the sugar and the duration of per-

FIG. 33.

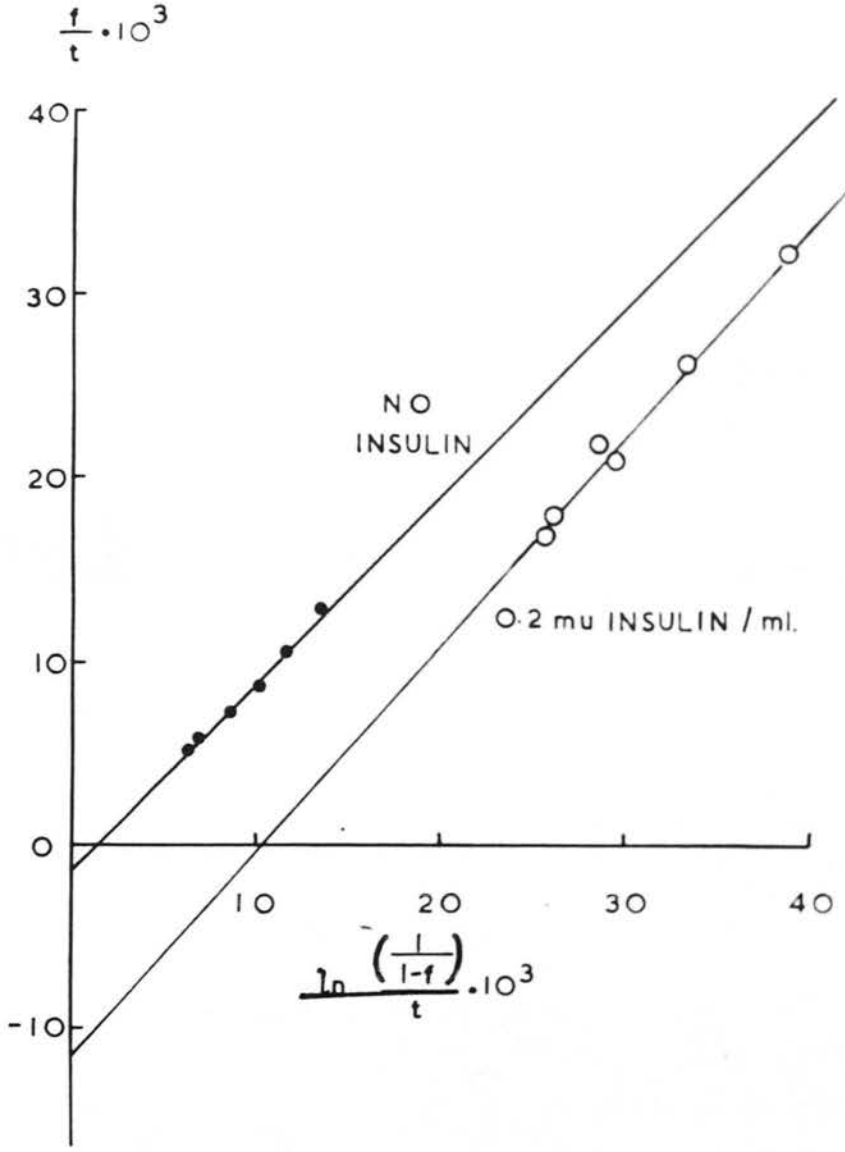
TIME-COURSE OF SUGAR UPTAKE



The points are the mean observed values ± SEM given in Tables 32 and 34. The curves through the points are based on the carrier transport system, using the determined values of K and V as in Tables 33 and 34.

FIG. 34.

PLOTS BASED ON PERMEATION EQUATION
FOR L-ARABINOSE IN PRESENCE AND ABSENCE OF INSULIN

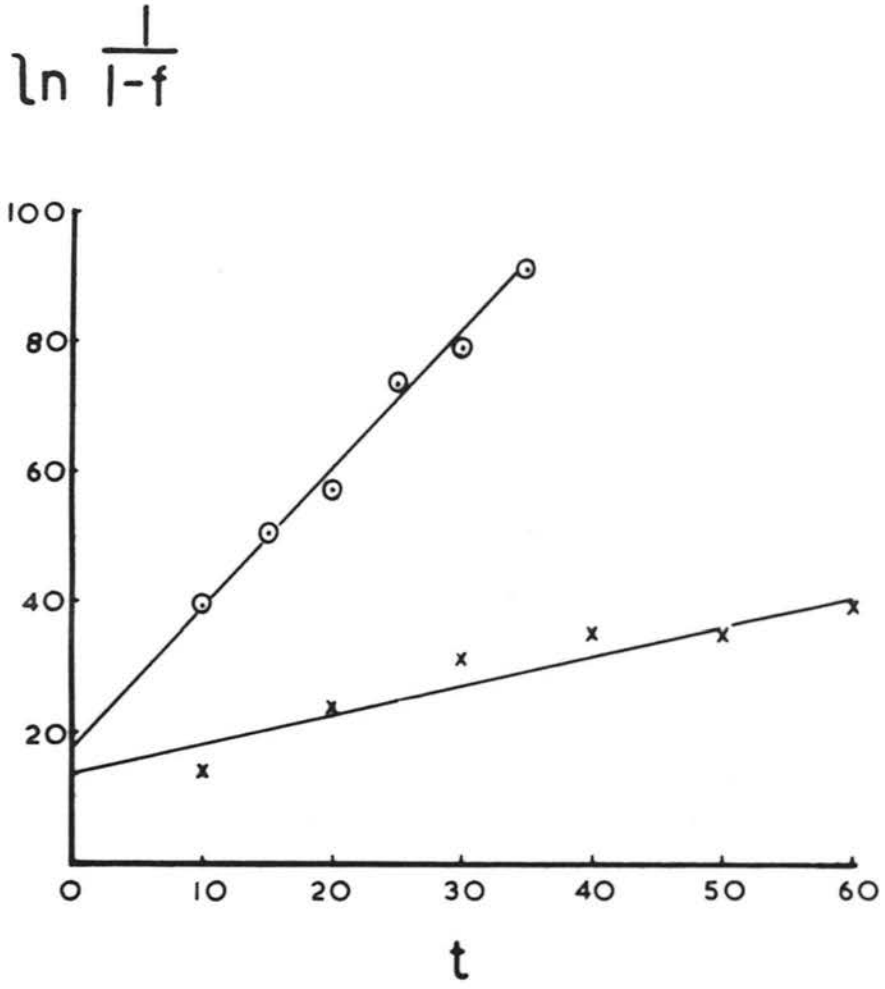


The values are given in Table 33.

The lines are regressions of each set of values.

FIG. 35.

TEST OF CONFORMITY OF ARABINOSE UPTAKE RESULTS TO A DIFFUSION TRANSPORT PROCESS



The points are calculated from the mean percentage penetration of cell water by arabinose at the times t given in Table 32.

The lines are regressions of each set of values.

fusion. Table 32 gives the results of these experiments which are illustrated in Fig. 33. Both in the presence and absence of insulin, there is a fall in the rate of transport of sugar as the perfusion time increases, and this is most pronounced over the first 20 min. of perfusion. Insulin undoubtedly stimulates sugar uptake in the perfused heart. Table 33 gives the values of $\frac{f}{t}$ and $\ln\frac{1}{1-f}/t$. calculated from the mean penetrations at different perfusion times, and also the values for the slope and the intercept of the regression line calculated from the $\frac{f}{t}$ and $\ln\frac{1}{1-f}/t$. results. At the foot of this Table are the derived values of K and V. Fig. 34 shows the fit of the points to the regression line, and it will be seen that the results conform well to the permeation equation both in the presence and absence of insulin. That is, in each case the slope of the regression line is greater than unity and the intercept on the $\frac{f}{t}$ axis is negative. The effects of insulin of increasing K and diminishing V as reported by Fisher and Zachariah (1961) were confirmed here. It should be mentioned that the curves through the points of Fig. 33 were calculated by substituting the values of K and V in the permeation equation. Another point which should be made on examining these sugar uptake results is that if the permeation process were one of simple diffusion, it would be expected that there would be a direct proportionality between $\ln\frac{1}{1-f}$ and t. Fig. 35 shows this not to be the case.

TABLE 32

Percentage Penetration of Cell Water in Presence and Absence of Insulin

Arabinose Concentration 9.25mM. No Insulin.

	Perfusion Time (min.)					
	10	20	30	40	50	60
	8.0	34.5	21.8	16.2	25.6	13.9
	16.4	23.3	29.5	35.7	19.3	23.7
	19.3	16.5	7.5	26.9	28.4	13.1
	15.8	18.9	13.8	28.3	16.7	35.3
	0.3	2.5	23.3	33.5	12.6	35.7
	25.7	32.7	37.7	48.2	44.5	32.1
		33.0	45.6	31.6	34.2	77.6
	4.5	5.5	37.4	12.0	50.7	25.1
Mean	12.9	20.9	27.1	29.1	29.0	32.1
SEM	±3.4	±4.4	±4.6	±4.0	±4.8	±7.2
Expts.	7	8	8	8	8	8

Table 32 (contd.)

Arabinose Concentration 10.50mM. Insulin concentration 0.2 mU/ml.

	Perfusion Time (min.)					
	10	15	20	25	30	35
	33.4	41.2	44.0	54.0	56.5	48.1
	30.6	52.8	72.1	48.0	46.0	54.1
	-	36.3	30.6	84.1	46.2	40.6
	24.3	35.0	49.1	26.2	59.7	62.2
	32.8	44.1	37.2	49.7	64.2	63.0
	40.0	27.4	27.7	49.9	53.8	89.3
Mean	32.2	39.5	43.5	52.0	54.4	59.6
SEM	±2.5	±3.6	±6.6	±7.6	±3.0	±6.9
Expts.	5	6	6	6	6	6

Hearts were preperfused on the basic medium for 40 min. prior to perfusion on the sugar-containing media for the times shown. Insulin, when used, was present in both preperfusion and perfusion media.

Where there is a dash, the percentage penetration result was considered unacceptable because of some defect in the perfusion or analytical techniques.

TABLE 33

Arabinose Results of Table 32 Converted to Values Used to Calculate K and V

No Insulin			Insulin 0.2 mu./ml.		
$\frac{f}{t}$. 10^3	$\ln \frac{1}{1-f}/t.$ 10^3	Regression Constants	$\frac{f}{t}$. 10^3	$\ln \frac{1}{1-f}/t.$ 10^3	Regression Constants
12.9	13.8	b = 1.00509	32.2	38.9	b = 1.12970
10.5	11.7		26.3	33.6	
9.0	10.5	a = -1.216	21.8	28.5	a = -11.640
7.3	8.6		20.8	29.4	
5.8	6.8	Se of b = $\pm$0.038	18.1	26.2	Se of b = $\pm$0.072
5.4	6.5		17.0	25.9	
K = 0.05mM V = 2.22mM/min			K = 1.36mM V = 1.06mM/min.		

The regression line depends on the equation derived in Section I.

$$\frac{f}{t} = \frac{K + x}{x} \cdot \ln \frac{1}{1-f}/t. - \frac{KV}{x(K + x)}$$

It is convenient to multiply the values of $\frac{f}{t}$ and $\ln \frac{1}{1-f}$ by 10^3 in presenting the results. The values of 'a' actually used to calculate the values of V are therefore the values given in the tables times 10^3

Time course of D-xylose uptake

Similar experiments to those described for L-arabinose were performed using D-xylose as the test sugar. A preliminary experiment indicated that at a xylose concentration of 10mM, in the presence of 0.2 mU. insulin/ml. xylose entered the heart cells too rapidly for the convenient measurement of sugar uptake. Use was therefore made of the inverse relationship between sugar uptake and the perfusate sugar concentration established in Section IV. It was found that if the xylose concentration of the perfusate was raised to the region of 30mM, the time course of xylose uptake could readily be studied in the presence of 0.2 mU. insulin/ml. perfusate. In the absence of insulin 10 mM xylose was used.

The results of these experiments are shown in Table 34. The standard errors of the means tend to be larger in the insulin series indicating the greater variability in that series. Comparatively little difference will be seen in the two sets of $\frac{f}{t}$ and $\ln \frac{1}{1-f}/t$ values computed from these results because of the different sugar concentrations used, but as for arabinose it will be seen that insulin markedly increases K and causes a diminution in V.

TABLE 34

Percentage Penetration of Cell Water by D-Xylose in the Presence and Absence of Insulin

Xylose Concentration 9.65mM. No Insulin

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t} \cdot 10^3$	$\ln \frac{1}{1-f}/t \ 10^3$	Regression Constants
10	18.3±2.5 (8)	18.3	20.2	b = 1.01607
20	27.0±3.2 (8)	13.5	15.7	
30	30.9±2.4 (7)	10.3	12.3	a = -2.33
40	37.6±3.4 (7)	9.4	11.8	
50	39.7±1.7 (8)	7.9	10.1	Se of b= ±0.020
60	41.8±5.7 (8)	7.0	9.0	

K = 0.16mM. V = 1.42mM/min.

Xylose Concentration 28.31mM. Insulin Concentration 0.2 mU/ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t} \cdot 10^3$	$\ln \frac{1}{1-f}/t \ 10^3$	Regression Constants
10	16.6 ± 4.3 (4)	16.6	18.2	b = 1.17397
20	25.4 ± 7.7 (7)	12.7	14.7	
30	30.1 ± 3.8 (7)	10.0	11.9	a = -4.87
40	42.3 ±10.7 (7)	10.6	13.7	
50	47.0 ± 9.1 (7)	9.4	12.7	Se of b = ±0.107
60	49.0 ± 7.6 (6)	8.2	11.2	

K = 4.93mM V = 0.93mM/min.

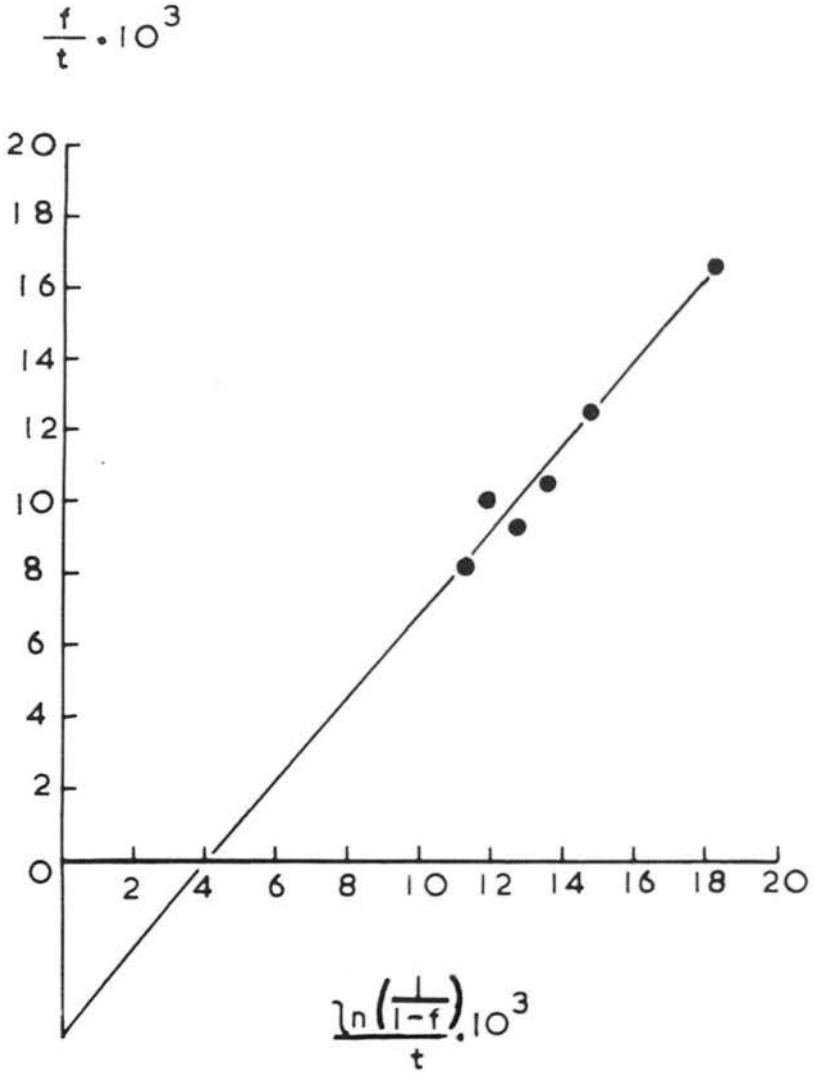
Hearts were preperfused for 40 min. on the basic medium and then perfused with the sugar-containing media for the times shown. Insulin, when used, was present in both preperfusion and perfusion media. Only the mean values are given.

Because of the overlap of these two sets of results it is confusing to illustrate both sets on one figure. The conformity of the non-insulin set to the permeation hypothesis is shown in Fig. 33 with the arabinose data. The curve through the points was calculated by substituting the values of K and V of Table 34 in the permeation equation. In Fig. 33 the curves for the two sugars may be compared because the sugar concentration involved in each case is approximately 10mM. The standard errors of the xylose points have been omitted for the sake of clarity, but Table 34 shows them to be smaller than those illustrated for arabinose. It should be noted that xylose penetrates the heart cells more rapidly than does arabinose at the same concentration, and that the value of K for xylose is considerably larger than that of arabinose.

It will be apparent that the fit of the sugar uptake results to the permeation equation can be illustrated either by the fit of the $\frac{f}{t}$ and $\ln \frac{1}{1-f}/t$ values to the regression line based on the equation, or by the fit of the percentage penetration results to the curve calculated from the parameters obtained from this

FIG. 36.

CONFORMITY OF XYLOSE INSULIN DATA TO PERMEATION EQUATION



The points are calculated from the mean percentage penetration of cell water by the sugar at time t as given in Table 34.

The regression line has been drawn through the points.

regression. The conformity of the xylose-uptake results obtained in the presence of insulin is shown in Fig. 36 by the second method. The results conform well to the equation.

Sugar uptake at higher insulin concentrations

Sugar penetration experiments were performed as described previously, with insulin present in the perfusion media in final concentrations of 1.0 mU./ml. and 4.0 mU./ml. in different experiments. At these high insulin concentrations, high sugar concentrations in the perfusate are essential to slow down the rate of transport. The results of arabinose uptake experiments are shown in Table 35. It will be seen that the K values obtained at these higher insulin concentrations are in reasonable agreement with the value obtained in the present of 0.2 mU. insulin/ml. The values of V, however, increase with the insulin concentration.

The results from similar experiments employing xylose are shown in Table 36. Again the values of K at the two higher insulin concentrations agree reasonably well with that found in the

TABLE 35

Percentage Penetration of Cell Water by L-Arabinose in the Presence of Insulin

Arabinose Concentration 30.00mM. Insulin Concentration 1.0 mU./ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t} \cdot 10^3$	$\ln \frac{1}{1-f}/t \cdot 10^3$	Regression Constants
10	18.5 ± 5.8 (6)	18.5	20.5	b = 1.03703
20	29.2 ± 8.3 (6)	14.6	17.3	
30	34.5 ± 8.5 (6)	11.5	14.1	a = -4.430
40	54.4 ± 3.3 (5)	13.6	19.6	
50	53.4 ± 9.7 (6)	10.7	15.3	Se of b = ±0.295
60	58.5 ± 8.7 (6)	9.8	14.7	

K = 1.11mM V = 3.72mM/min.

Arabinose Concentration 50.12mM. Insulin Concentration 4.0 mU/ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t} \cdot 10^3$	$\ln \frac{1}{1-f}/t \cdot 10^3$	Regression Constants
10	43.4 ± 7.3 (5)	43.4	56.9	b = 1.03763
15	50.0 ± 3.4 (6)	33.3	46.2	
20	65.2 ± 5.3 (5)	32.6	52.8	a= -19.025
25	69.6 ± 6.1 (6)	27.8	47.6	
30	67.8 ± 6.9 (6)	22.6	37.8	Se of b = ±0.286
35	78.8 ± 9.3 (6)	22.5	44.3	

K = 1.89mM V = 26.3mM/min.

Hearts were preperfused for 40 min. on the basic medium and then perfused with the arabinose-containing media for the times shown. Insulin, when used, was present in both preperfusion and perfusion media. Only the mean values are shown in the Table.

TABLE 35

Percentage Penetration of Cell Water by L-Arabinose in the Presence of Insulin

Arabinose Concentration 30.00mM. Insulin Concentration 1.0 mU./ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t} \cdot 10^3$	$\ln \frac{1}{1-f}/t \cdot 10^3$	Regression Constants
10	18.5 ± 5.8 (6)	18.5	20.5	b = 1.03703
20	29.2 ± 8.3 (6)	14.6	17.3	
30	34.5 ± 8.5 (6)	11.5	14.1	a = -4.430
40	54.4 ± 3.3 (5)	13.6	19.6	
50	53.4 ± 9.7 (6)	10.7	15.3	Se of b = ±0.295
60	58.5 ± 8.7 (6)	9.8	14.7	

K = 1.11mM V = 3.72mM/min.

Arabinose Concentration 50.12mM. Insulin Concentration 4.0 mU/ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t} \cdot 10^3$	$\ln \frac{1}{1-f}/t \cdot 10^3$	Regression Constants
10	43.4 ± 7.3 (5)	43.4	56.9	b = 1.03763
15	50.0 ± 3.4 (6)	33.3	46.2	
20	65.2 ± 5.3 (5)	32.6	52.8	a= -19.025
25	69.6 ± 6.1 (6)	27.8	47.6	
30	67.8 ± 6.9 (6)	22.6	37.8	Se of b = ±0.286
35	78.8 ± 9.3 (6)	22.5	44.3	

K = 1.89mM V = 26.3mM/min.

Hearts were preperfused for 40 min. on the basic medium and then perfused with the arabinose-containing media for the times shown. Insulin, when used, was present in both preperfusion and perfusion media. Only the mean values are shown in the Table.

TABLE 36

Percentage Penetration of Cell Water by D-Xylose in Presence of Insulin

Xylose Concentration 54.40mM. Insulin Concentration 1.0 mu/ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t}$. 10^3	ln $\frac{1}{1-f}/t$. 10^3	Regression Constants
10	20.6 ± 6.0 (5)	20.6	23.1	b = 1.10146
20	34.2 ± 12.7 (5)	17.1	20.9	
30	50.1 ± 13.1 (4)	16.7	23.2	a = -6.690
40	50.4 ± 5.7 (5)	12.6	17.5	
50	57.7 ± 9.9 (5)	11.5	17.2	Se of b = ±0.196
60	58.5 ± 10.8 (4)	9.8	14.7	

K = 5.52mM. V = 3.95 mM/min.

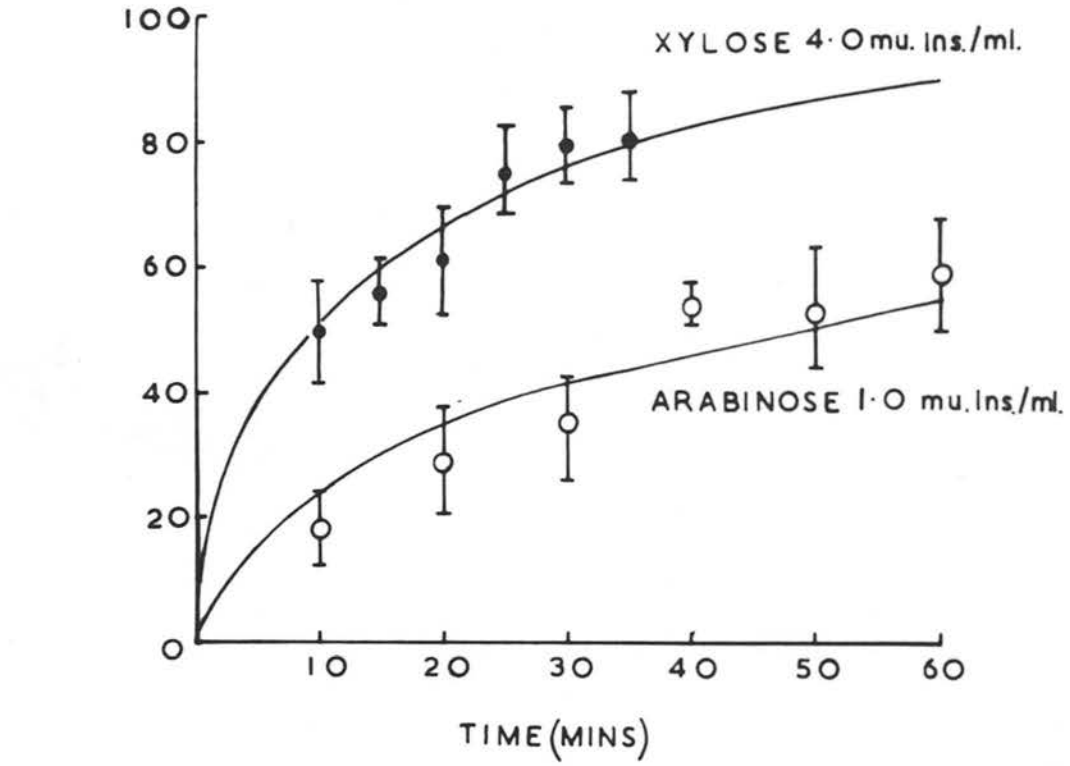
Xylose Concentration 69.01mM. Insulin Concentration 4.0 mu./ml.

Perfusion Time t (min.)	Mean Penetration ± SEM and No. of Expts.	$\frac{f}{t}$. 10^3	ln $\frac{1}{1-f}/t$. 10^3	Regression Constants
10	50.0 ± 8.1 (6)	50.0	69.3	b = 1.08159
15	56.4 ± 5.2 (6)	37.6	55.3	
20	61.3 ± 8.7 (6)	30.7	47.5	a = -26.220
25	75.4 ± 7.2 (6)	30.2	56.1	
30	79.4 ± 5.9 (6)	26.5	52.6	Se of b = ±0.266
35	81.3 ± 6.7 (5)	23.2	47.9	

K = 5.63mM. V = 24.0mM./min.

FIG. 37.

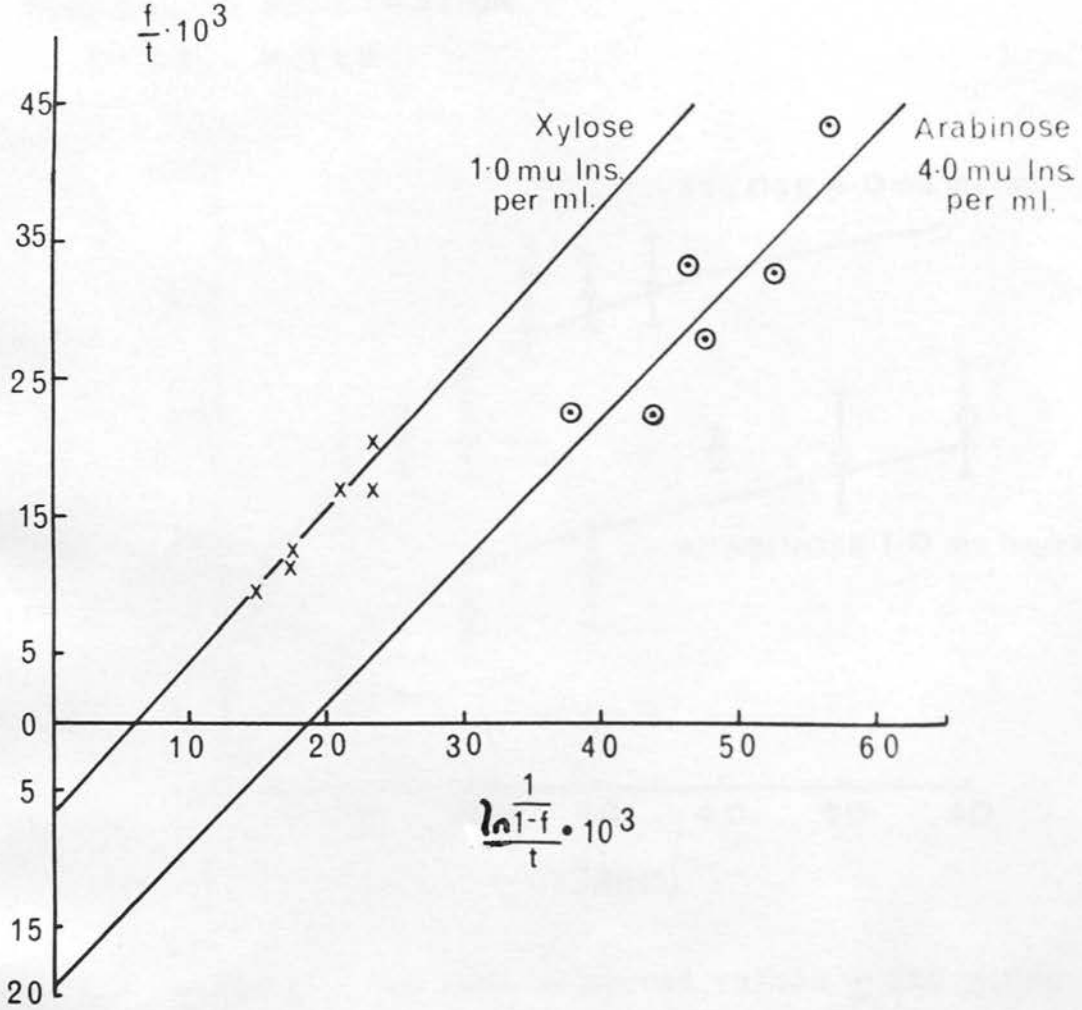
SUGAR UPTAKE AT HIGH INSULIN CONCENTRATIONS



The points are the mean observed values ± SEM given in Tables 35 and 36. The curves through the points are based on the carrier transport system using the determined values of K and V given in these tables.

FIG. 38.

Conformity of Sugar Uptake Results to Permeation Equation at High Insulin Concentrations



The points are calculated from the mean percentage penetration of cell water by the sugar as given in Tables 35 and 36.

presence of 0.2 mU. insulin/ml. perfusate. The values of V tend to increase as the perfusate insulin concentration is increased. The fit of the arabinose and xylose results to the permeation equation is shown in Fig. 37 and Fig. 38. The results are most conveniently illustrated by putting the arabinose and xylose curves together in this fashion because of the overlap of the results at the two insulin concentrations for any one sugar. The form of the curves of Fig. 37 is the same as that found at the low insulin concentration and in the absence of insulin. At these higher insulin concentrations, however, the results do not fit the permeation equation so closely as previously observed, and the greater variability of these last results is indicated by the standard errors of the slopes of the regression lines. These are very much larger than those found at the insulin concentration of 0.2 mU./ml. or those found when no insulin is present in the perfusion media. In this connection it has to be borne in mind that at high values of f, the rate of increase of $\ln \frac{1}{1-f}$ is very great relative to that of f itself so that an unattainable degree of experimental precision would be necessary if the variability was to be left within desirable limits.

The osmotic correction

The values of the permeation parameters, and V, so far presented have been calculated using the permeation equation

$$\frac{f}{t} = \frac{K + x}{x} \quad \frac{\ln \frac{1}{1-f}}{t} \quad - \quad \frac{KV}{x(K + x)}$$

To allow for the widely differing perfusate sugar concentrations employed in these sugar-uptake experiments, K and V have been recalculated on the basis of the osmotically corrected permeation equation derived in Section I.

$$\frac{f}{t} = \left(\frac{m+x}{K+m+x}\right) \quad \frac{K + x}{x} \quad \ln \frac{1}{1-f}/t^{-} \left(\frac{m}{K+m+x}\right) \quad \frac{KV}{x(K+x)}$$

The results are presented in Table 37 where Ko and Vo are the values obtained after applying the osmotic correction. It will be seen that for both xylose and arabinose, the corrected and uncorrected values are very similar at the low insulin concentration and in the absence of hormone. This is because of the low sugar concentrations used in the perfusates under these conditions. At high insulin concentrations, however, high sugar concentrations have been used, and the osmotic correction has a correspondingly greater effect. For both sugars the K values

TABLE 37

Values for the Parameters of Sugar Transport in the Presence and Absence of Insulin

The values are calculated on the basis of the permeation equations in the osmotically corrected and uncorrected forms as described in Section IV.

Insulin Concentration (mU./ml.)	D (+) Xylose					L (-) Arabinose				
	Concn. (Millimolar)	Apparent Dissociation Constant (Millimolar)		Maximal Transport Rate (Millimoles/min.)		Concn. (Millimolar)	Apparent Dissociation Constant (Millimolar)		Maximal Transport Rate (Millimoles/min.)	
		K	Ko	V	Vo		K	Ko	V	Vo
0.0	9.65	0.16	0.17	1.42	1.47	9.25	0.05	0.05	2.23	2.16
0.2	28.31	4.93	5.49	0.93	1.03	10.50	1.36	1.41	1.06	1.11
1.0	54.40	5.52	6.69	3.95	4.75	30.00	1.11	1.23	3.72	4.12
4.0	69.01	5.63	7.08	24.00	30.06	50.12	1.89	2.23	26.30	30.87

K and V are the uncorrected parameters

K_o and V_o are the osmotically corrected parameters

tend to increase slightly as the insulin concentration changes, all values being far higher than that found in the absence of hormone. Where V is concerned, the agreement between the values obtained for the two sugars at any one insulin concentration should be noted. After the initial fall in V when 0.2 mU. insulin/ml. of perfusate is added, the values increase steadily as the insulin concentration is increased and in view of the theoretical considerations outlined in the Introduction, this is a surprising finding. Indeed, these results indicate a relationship of direct proportionality between the magnitude of V and the insulin concentration. This was further tested, for both sugars, by perfusing 6 hearts with a perfusate containing a high sugar concentration and an insulin concentration of 20.0 mu./ml.

The results are given in Table 38. No accurate uptake studies could be performed at this high insulin concentration, so the values of V have been calculated assuming that for each sugar K has the mean value of those determined at the other insulin concentrations. The calculated value of V is seen to increase well above the

TABLE 38

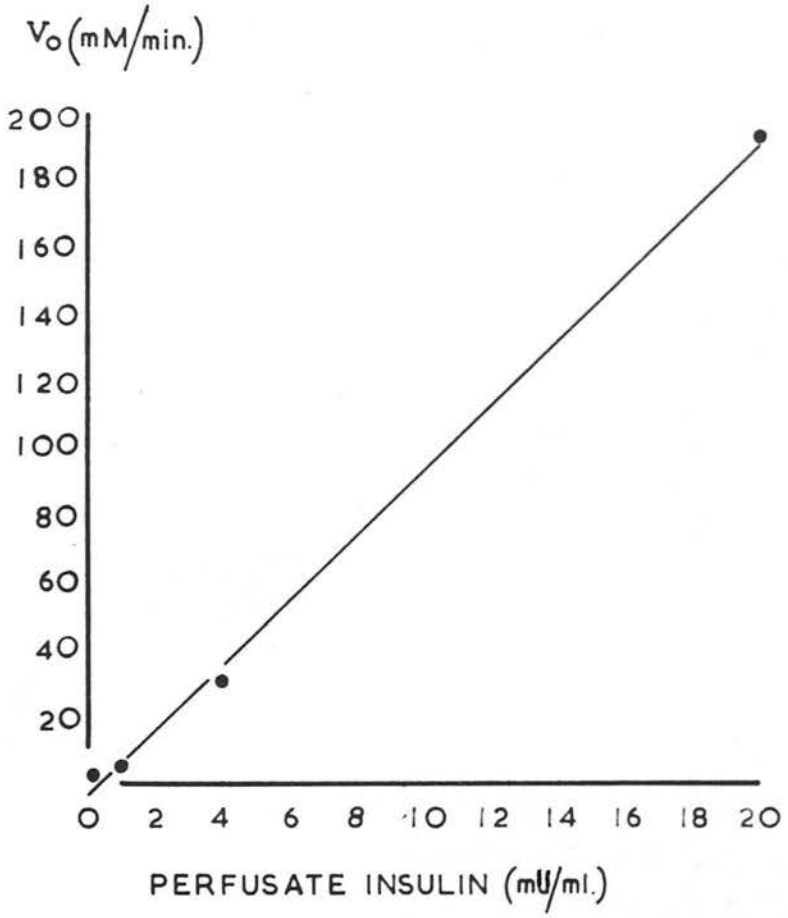
Estimates of V at an Insulin Concentration of 20.0 mu./ml.

Sugar	D (+) Xylose				L (-) Arabinose			
Concentration	104.5mM				66.2mM			
Mean Percentage Penetration (± SEM)	76.6 ± 3.2 (6)				65.9 ± 2.6 (6)			
Assumed Values of K (mM) and calculated values of V (mM/min.)	K	Ko	V	Vo	K	Ko	V	Vo
	5.36	6.42	163.0	217.7	1.45	1.62	136.0	165.6

For each sugar, 6 hearts were perfused for 10 min. on the sugar-containing medium with insulin after the standard preperfusion period. The values of K are the means of those found at the lower insulin concentrations as given in Table 37. The values of V were calculated by using the permeation equation and the osmotically corrected permeation equation (Section I p. 35) after substituting the values of K, x, t and f. K_o and V_o are the osmotically corrected parameters.

FIG. 39.

RELATIONSHIP BETWEEN OSMOTICALLY CORRECTED V_o AND PERFUSATE INSULIN CONCENTRATION



The average values of V from the xylose and arabinose results at any insulin concentration have been osmotically corrected. The values of V_o so obtained, which are plotted above, are given in Tables 37 and 38.

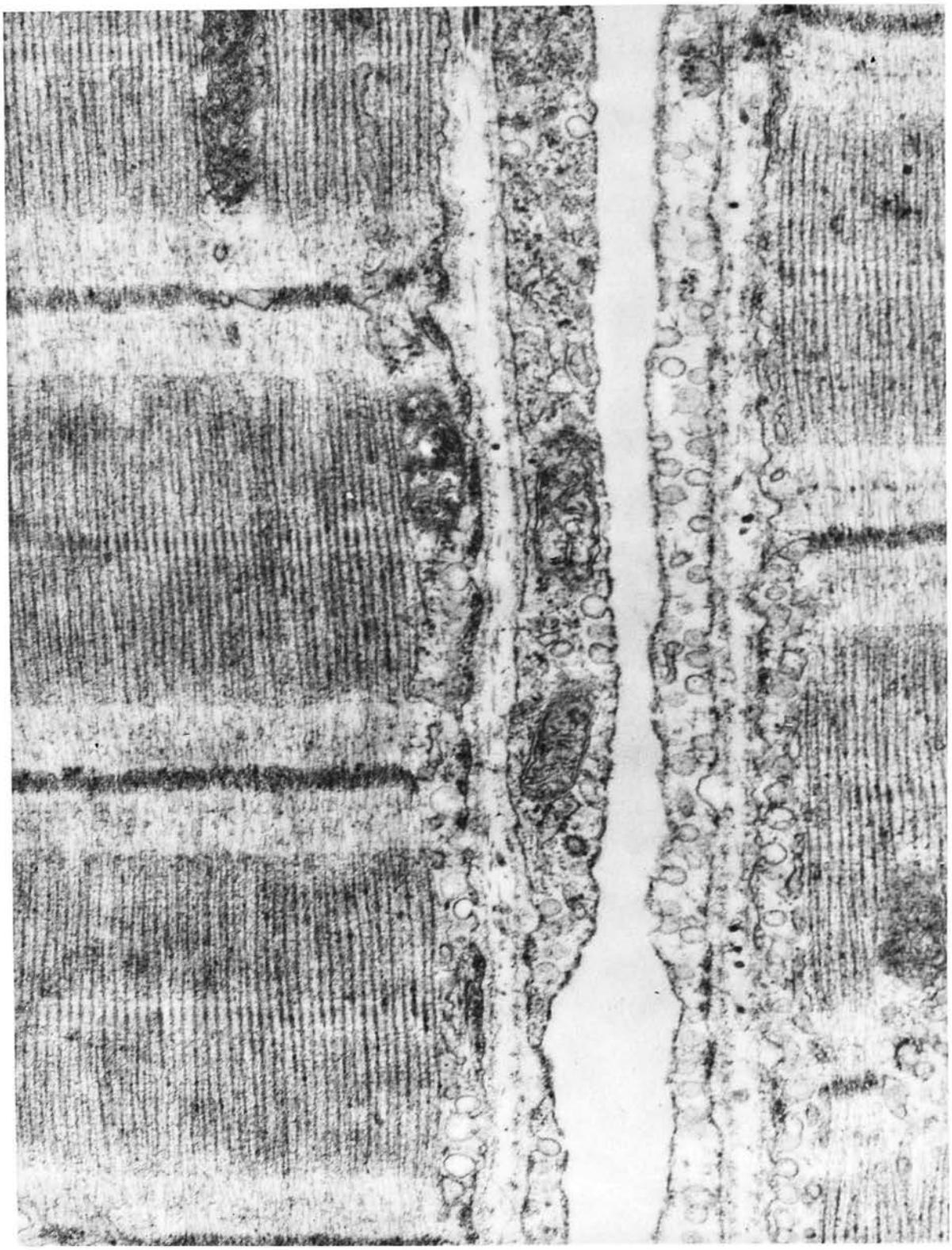
values obtained at the other insulin concentrations. Because of the extremely high perfusate sugar concentrations necessary to prevent 100% penetration of the cell water by the sugar when this very high insulin concentration is used, the application of the osmotic correction results in a considerable change in V. A plot of the osmotically corrected values of V at the different perfusate insulin concentrations is given in Fig. 39. There does seem to be a relationship of approximate proportionality between the maximal transport rate and the insulin concentration.

Pinocytosis and sugar transport

A consideration of the possible mechanisms by which V could increase in proportion to the insulin concentration of the perfusate led to the interesting possibility that the process of pinocytosis might be involved in sugar transport, and this point is discussed later. Experiments were therefore performed to test this possibility.

A heart was perfused for 15 min. on the basic medium. At the end of this time, 5 ml. of a solution of 20% formalin in veronal buffer

FIG. 40.

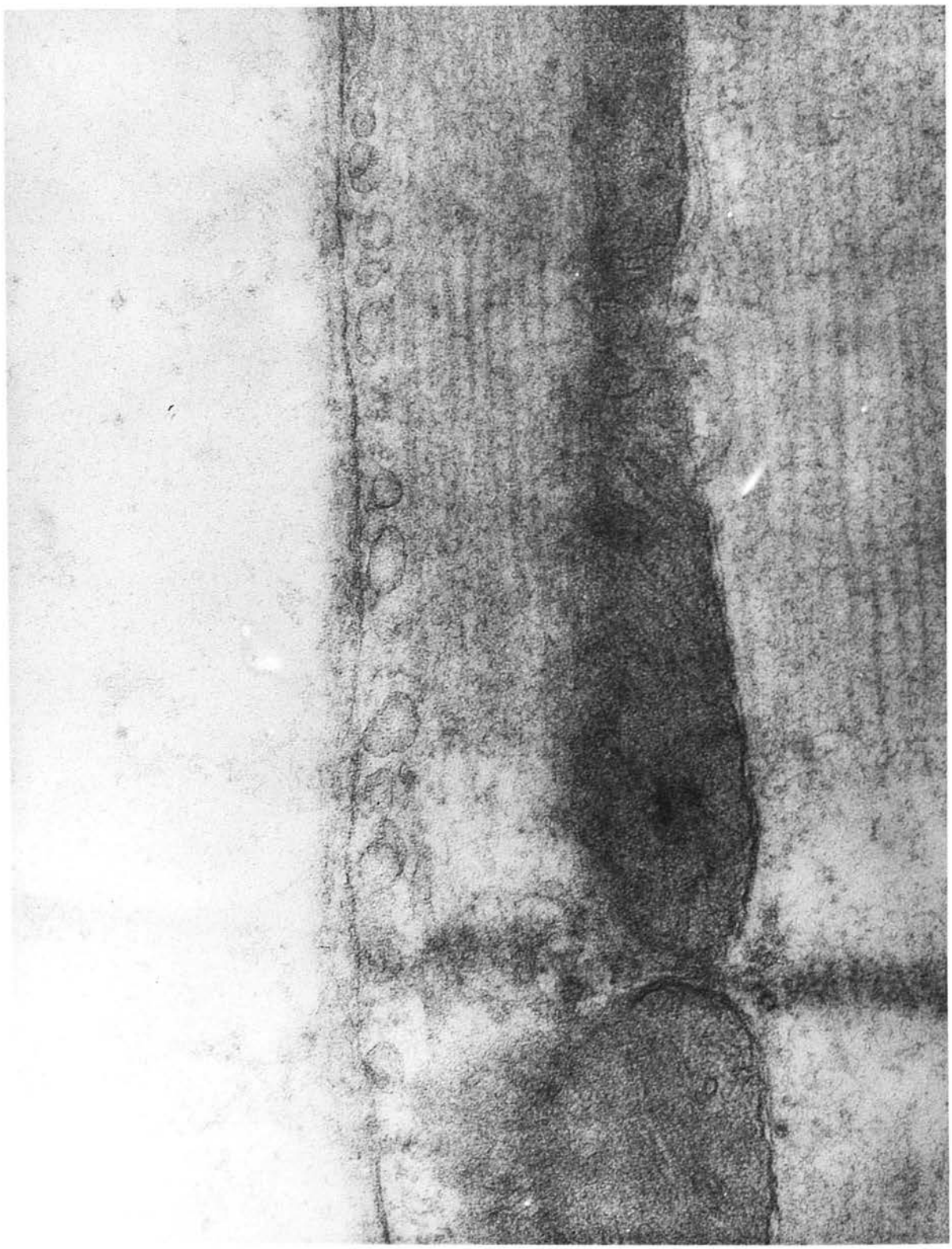


Electron micrograph of section of rat ventricle showing vescicles in capillary wall and myocardial cell wall Formalin fixation x 57,000.

pH 7.4 was injected into the rubber tube leading to the cannula bearing the heart. The heart was then cut from the cannula and allowed to stand in a vessel containing the 20% formalin fixative for 30 min. It was then treated as described under methods to prepare electron micrographs. One of these is shown in Fig. 40. It will be seen that vesicles are present in both endothelial membrane of the capillary and in the membranes of the cardiac muscle cells.

The possible implication of pinocytosis in sugar transport under the conditions of our experiments was tested as follows. Three hearts were perfused for 40 min. on the basic medium and then transferred to a similar medium containing 10mM arabinose for 10 min. The preperfusion and perfusion media for one of the hearts contained 0.2 mU insulin/ml. and for another 20.0 mU insulin/ml. The third heart was perfused in the absence of insulin. The hearts were fixed with osmium tetroxide and electron micrographs prepared from them as described under Methods. Counts were made of the numbers of pinocytotic vesicles occurring along 7.5 μ lengths of the cell membrane under each set of conditions.

FIG. 41.



Electron micrograph of section of ventricle of rat perfused with 10mM Arabinose in the absence of insulin. Osmium fixation x 144,000.

Seven randomly chosen prints were examined for each heart. Table 39 shows the results of the counts, and Fig. 41 shows a typical portion of cell membrane from which such counts were made. It will be seen that insulin does not exert a statistically significant effect upon the number of vesicles in the cell membrane.

TABLE 39

Counts of Pinocytotic Vesicles in Perfused Hearts

Figures represent average number of vesicles per 7.5 μ length of membrane.

Arabinose Concn.(mM)	10	10	60
Insulin Concn. (mU./ml.)	0	0.2	20.0
	3.00	5.85	7.06
	5.00	2.81	2.50
	9.00	4.71	8.34
	2.00	2.41	8.00
	3.25	14.00	6.43
	5.00	9.75	12.19
	2.57	4.69	9.00
Mean ± SEM	4.26 ± 0.90	6.32 ± 1.57	7.65 ± 1.11

Analysis of Variance of the Above Data

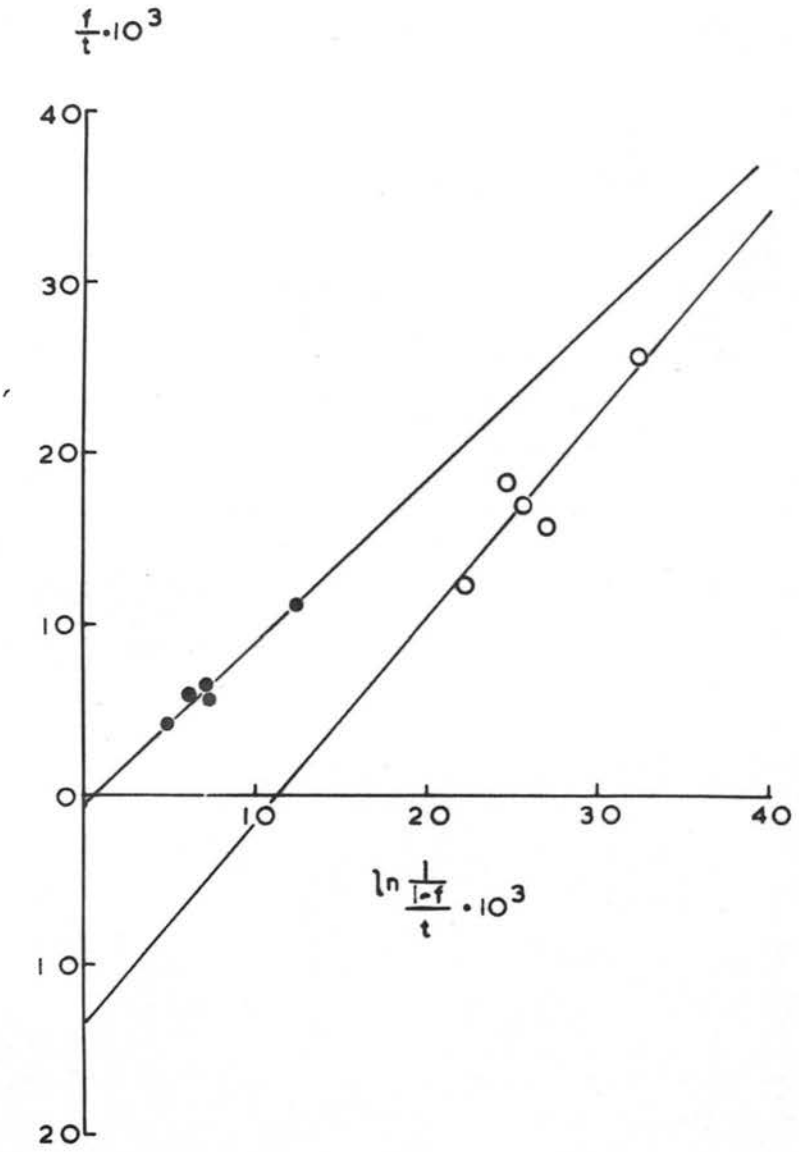
Source of Variation	Deg. Freedom	Sums of Squares	Mean Square	Variance Ratio	Probability
Total	20	230.0911			
Dosage	2	40.7400	20.3700	1.94	0.2>P>0.1
Residual	18	189.3511	10.5195		

The hearts were preperfused for 40 min. on the basic medium prior to perfusing for 10 min. on sugar containing media. Insulin, when used, was present in both preperfusion and perfusion media. Electron micrographs were prepared after osmium fixation.

FIG. 42.

PLOTS BASED ON PERMEATION EQUATION FOR L-ARABINOSE IN THE PRESENCE AND ABSENCE OF INSULIN

DATA OF FISHER AND ZACHARIAH



The points are calculated from the mean fractional penetration (f) of cell water by the sugar at time t as given in the paper of Fisher and Zachariah (1961) The lines are regressions of each set of observations.

CHAPTER IV

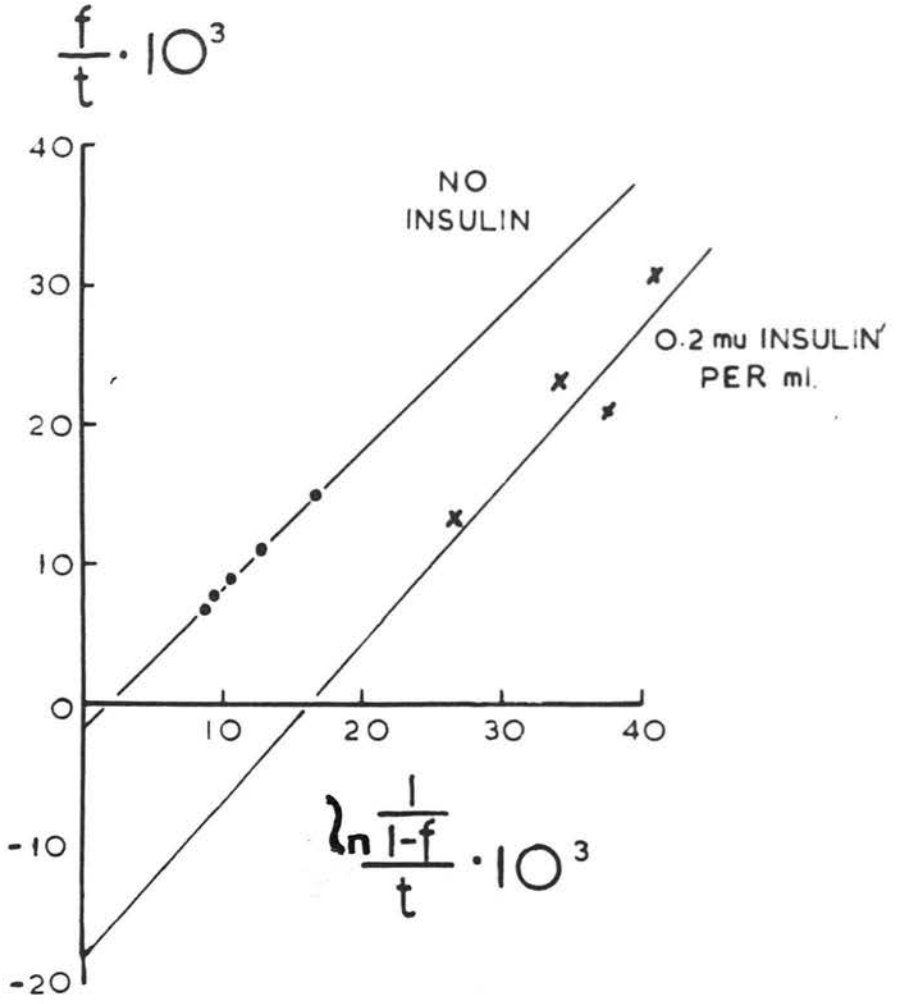
Discussion

Conformity of data to the permeation equation

The basis of all the experiments performed in this section has been the permeation equation developed in Section I from the carrier transport of Widdas (1952, 1954). The results of the sugar uptake experiments conform well to this equation both in the presence and absence of insulin. In order to compare the results with those of Fisher and Zachariah (1961) it is necessary to re-examine the data obtained by those workers, for their sugar uptake results were not directly tested by the plot of $\frac{f}{t}$ and $\ln \frac{1}{1-f/t}$ values used here. This has now been rectified, and the plot so obtained from the uptake results for L-arabinose is shown in Fig. 42. The fit of the points to the regression line is quite reasonable both in the presence and absence of insulin. However, the slope, b, of the line in the absence of hormone is 0.947 ± 0.025. Since by our treatment K is obtained from this by applying the expression $K = x\,(b-1)$, then the value of K will be negative, and as such

FIG. 43.

PLOTS BASED ON PERMEATION EQUATION
D-XYLOSE UPTAKE IN PRESENCE AND ABSENCE
OF INSULIN DATA OF FISHER AND ZACHARIAH



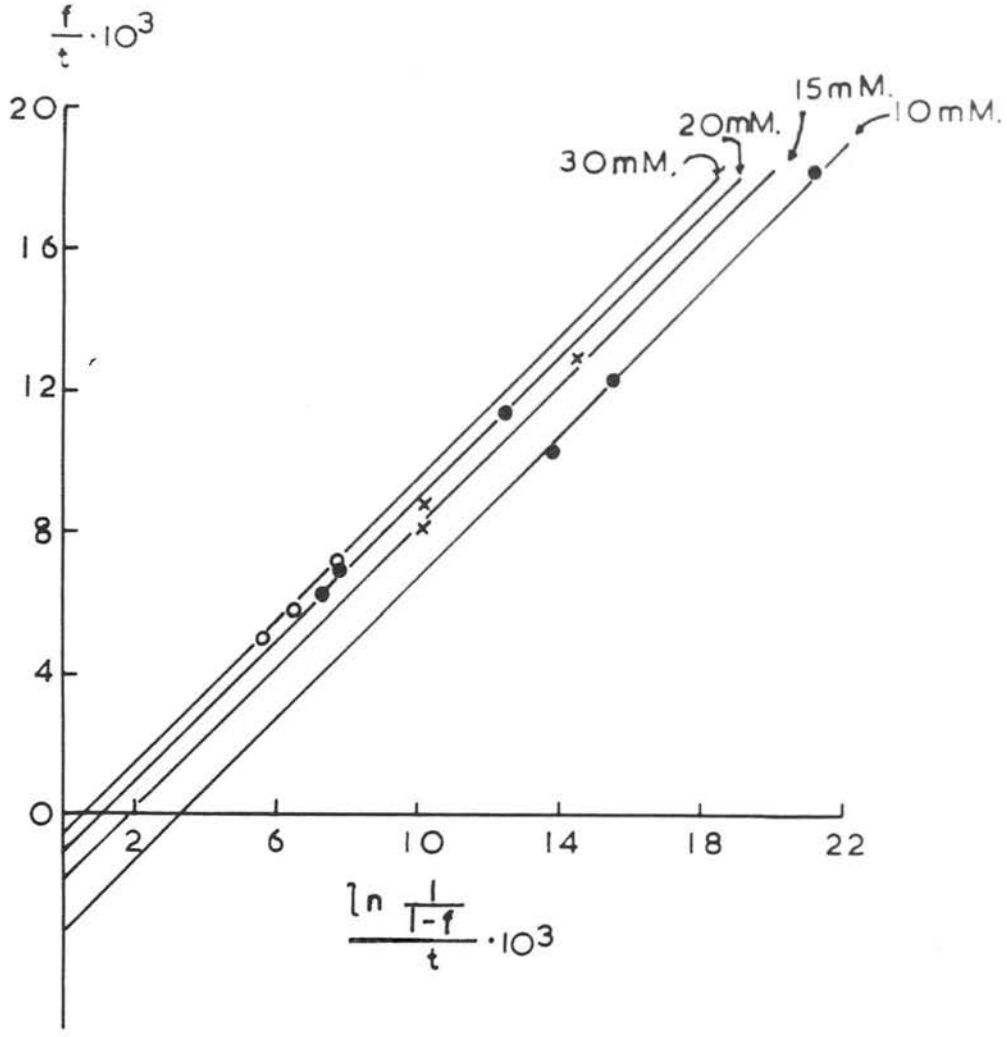
The points are calculated from the mean fractional penetration (f) of cell water by xylose at a time t, as given in the paper of Fisher and Zachariah (1961). The lines are regressions based on each set of observations.

unacceptable. It can at least be speculated that the true result for K will be very small and therefore not inconsistent with the value of 0.05mM obtained in the present work. When insulin is present, the slope of the regression line equals 1.181 ± 0.316, and since x = 30mM, then K = 5.43mM. This is higher than the value of 1.36 obtained in the present work, but not unreasonably so.

The plots of the xylose uptake data of Fisher and Zachariah are shown in Fig. 43. In the absence of insulin, the slope of the regression line is 0.978 ± 0.015 so that again K will be negative. From the standard error, however, the upper limit of b may be taken as 1.008, indicating that K is unlikely to exceed 0.24mM. The value found in the present work is 0.16mM. In the presence of insulin, the results of Fig.43 give a slope of b = 1.070 ± 0.322 indicating that K = 2.1mM. Again this agrees reasonably well with the value found here of 4.9mM. Where the values of V are concerned, it is difficult to speculate with estimates in the absence of insulin since V is calculated from $V = \frac{abx}{b-1}$ and b is smaller than unity for both sugars. In the

FIG. 44.

CONFORMITY OF EARLIER DATA TO PERMEATION EQUATION



The points are calculated from the mean percentage penetration of cell water by the sugar at time t as given in Table 29 Section IV.

The lines through the points are based on the more precisely determined K value.

presence of insulin, for arabinose V = 0.26mM/min. and for xylose V = 0.71mM/min. These are not inconsistent with the results of 1.06mM/min. for arabinose and 1.93mM/min. for xylose found in the present investigation.

Further support for the method of treatment of the results is provided by examining the data obtained in Section IV where sugar uptake at three different perfusion times was studied with four different perfusate sugar concentrations. This data was inadequate for the reliable estimation of K and V directly because of the errors involved in the estimation when there are so few observations at any one sugar concentration. However, having calculated the constants for arabinose in a more precise fashion in this final section, the conformity of the earlier data to the permeation hypothesis can now be tested as shown in Fig. 44. Thus a line of slope equal to unity will be compatible with the value found for the apparent equilibrium constant for arabinose, (K = 0.05mM; actual slope = 1.00509) and such a line has been drawn through the points obtained at each of the four different sugar concentrations. The closeness of fit of the points

in each case indicates the conformity: (i) to the basic hypothesis itself, since the plot of $\frac{f}{t}$ by $\ln \frac{1}{1-f}/t$ should yield a straight line, and (ii) to the particular K value. In every case the agreement is good. This test can be taken a step further by calculating the values for V given by the intercepts of the lines. These are, 10mM - 6.4; 15mM - 7.7; 20mM - 8.0 and 30mM - 9.0mM/min. They are all of the same order as the estimate of 2.23mM/min. found with the more precise measurement.

Another point emerging from this examination of the earlier data is that the same kinetics apply to sugar uptake at all the perfusate sugar concentrations studied. This agrees with the results of this last section, and justifies the use made of high perfusate sugar concentrations to make measurements of K and V at high insulin concentrations. Use is thus made of the inverse relationship between the rate of sugar uptake and its perfusate concentration to counteract the stimulatory effect of insulin on the uptake. At low sugar concentrations and high insulin concentrations rate measurements would be of little value for the estimation of K and V by our technique.

Reliability of results

In spite of the fact that all the data tested conform well to the permeation equation a disquieting feature of the results will be apparent. For the estimation of K we take $K = x(b-1)$. We are therefore interested in the reliability of the excess of b over unity. In all cases, even where the coefficient of variation of b is only a few per cent, the standard error is of much the same magnitude as the excess of b over unity (see, for example, Table 34). As a consequence, it cannot be claimed that the values of K are determined with any great precision, and the slight rise in the values as the insulin concentration of the medium is increased is not significant. Here, then, is an inherent limitation of the technique, and it can be in part removed only by performing an impracticably large number of experiments. Even so, the values found for K and V are certainly the most likely values within the range indicated by the standard errors, and the order of magnitude is reliable. In this connection there are few values in the literature with which to compare the ones established here. The compatibility of the data of

Fisher and Zachariah has already been demonstrated. Unpublished experiments by Dr. Ivan Bihler of this department on the transport of L-arabinose into the cells of the perfused rabbit heart (personal communication) give a result for the product K V of 0.067 mM^2/min. in the absence of insulin. In the present work, using the perfused rat heart, the value for the product K V for arabinose in the absence of insulin is 0.11 mM^2/min (see Table 33, p.104). Other data for the rabbit heart are not yet available, but the agreement between the values given above at least increases the feasibility of the results of the present work.

Comparison of results with values obtained for erythrocytes

It is interesting to compare the values of K found here with those given by other workers for the transport process involving these sugars in the erythrocyte. Thus, the values found here in the absence of insulin, 0.16mM for xylose and 0.05mM for arabinose, differ greatly from the values of 21mM and 86mM, respectively, given by Wilbrandt (1961) and from the values of 48mM and

213mM for xylose and arabinose respectively given by Le-Fevre (1962). The differences in the tissues studied can account for the discrepances between the results for erythrocytes, and those for cardiac muscle cells, and it should be remembered in this connection that erythrocytes do not respond to insulin. There is an interesting possibility in comparing the apparent affinities of the carriers for the two tissues. The values for the erythrocyte are far higher than those for the muscle cell, even when insulin is present (K_o= 6.4mM for xylose and 1.6mM for arabinose). It has been suggested that the stimulation of sugar transport in the heart cells when insulin is present results from a diminution in the apparent affinity of a membrane carrier molecule for sugar. It is possible, therefore, that the affinity of the carrier for sugar in the erythrocyte is already at a very low level, as indicated by the magnitude of K, and that insulin thus fails to reduce the affinity further. A recent paper by Rieser (1963) is worthy of mention here. Rieser purports to show that treatment of erythrocytes with the proteolytic enzyme chymotrypsin results in the erythrocytes becoming

sensitive to insulin with respect to the glucose transport system. The treatment did not seem to non-specifically alter the permeability properties of the cells since insulin had no effect on the penetration of urea, glycerol or fructose. It can be speculated that a carrier in the red cell membrane with very low affinity for sugars is converted by chymotrypsin treatment to one with very high affinity by the splitting off of a protein moiety. It would be interesting, therefore, to measure the values of K and V in the erythrocyte after chymotrypsin treatment.

Validity of the values of Vmax.

Where V is concerned, some idea of the validity of the estimates is to be obtained by comparing them with figures given by other workers for glucose uptake by the heart under different conditions.
The average value for xylose and arabinose found here in the insulin free system (1.82mM/min.) is reasonably close to that found by Morgan et al. (1961) but their result was obtained from hearts which probably retained endogenous insulin activity.

Taking the intracellular water of the heart per gram true solids as being 3.2 ml./g. then from the value of 1.82mM/min. it can be calculated that the maximum rate of glucose uptake possible, in the absence of insulin, should be 63 mg./g. dry weight/hr. The assumption is made here that glucose is transported by the same system as the non-metabolised pentoses. This result is in excess of the figure of 15 mg./g. dry weight/hr. reported by Bleehen and Fisher, (1954) for the glucose uptake of the perfused heart, but it is not unreasonably higher. On the other hand, from the graph of V values at different insulin concentrations of the perfusion medium (Fig. 39), the maximal transport rate at 2 mu. insulin/ml. perfusate corresponds to an uptake of 390 mg. glucose/g. dry weight/hr. and quite clearly, far higher uptakes should be possible at higher insulin concentrations. Bleehen and Fisher (1954) found that at the insulin concentration of 2 mU/ml. the glucose uptake of the heart was 44 mg./g. dry weight/hr. and at a concentration of 20 mU./ml. it was 49 mg./g. dry weight/hr.

The workers concluded that increasing the medium insulin concentration above 2 mU/ml. did not significantly increase glucose uptake, and this is very different from what one might expect on the basis of the increase in V with the increase in insulin concentration in the present work.

There appear to be serious discrepancies, therefore, between the observed rates of glucose uptake by the heart in the presence and absence of insulin and the theoretical uptakes possible on the basis of the values of V found in the present work. These discrepancies can be explained, however, in the following way. There is good evidence that in the isolated heart perfused in the manner used here there is a limitation of the capacity for oxygen consumption. This limitation is independent of the nature of the nutrient. Fisher and Williamson (1961) showed that the Qo_2 of the heart is 39 $\pm$ 0.9 (8 observations) ml./g. dry weight/hr. whatever the nutrient offered. It is significant that the maximum rate of glucose uptake observed in the presence of insulin (49 mg./g. dry weight/hr: 2 observations, Bleehen and Fisher, 1954) corre-

sponds to a Qo_2 of 35 ± 0.7 ml./g. dry weight/hr. Thus approx. 90% of the oxygen is here used to oxidise glucose. If acetoacetate is the nutrient provided, it is oxidised at a rate corresponding to utilisation of all the oxygen. If acetoacetate, glucose and insulin is provided, there is no increase in Qo_2, and glucose uptake is markedly depressed below the level found in the absence of acetoacetate (Krebs and Williamson, 1961). The limitation on glucose uptake appears therefore to be a metabolic one, and would appear to be independent of membrane permeability. This point should also be considered in relation to the work done by the perfused heart. Bleehen and Fisher showed that if the tip of the cannula was pushed through the aortic valves during cannulation of the heart, the glucose uptake of the heart in the presence of insulin could be as high as 77 mg./g. dry weight/hr. The effect of the valvular incompetence would be to cause the heart ventricle to contract against a resistance, and the result of performing this work was possibly to alter the level of the metabolic constraint on the glucose uptake.

In view of these considerations, and since the results of V found here are more than adequate to account for the results of glucose-uptake obtained by other workers, these last mentioned results do not throw doubt on the validity of the values of V.

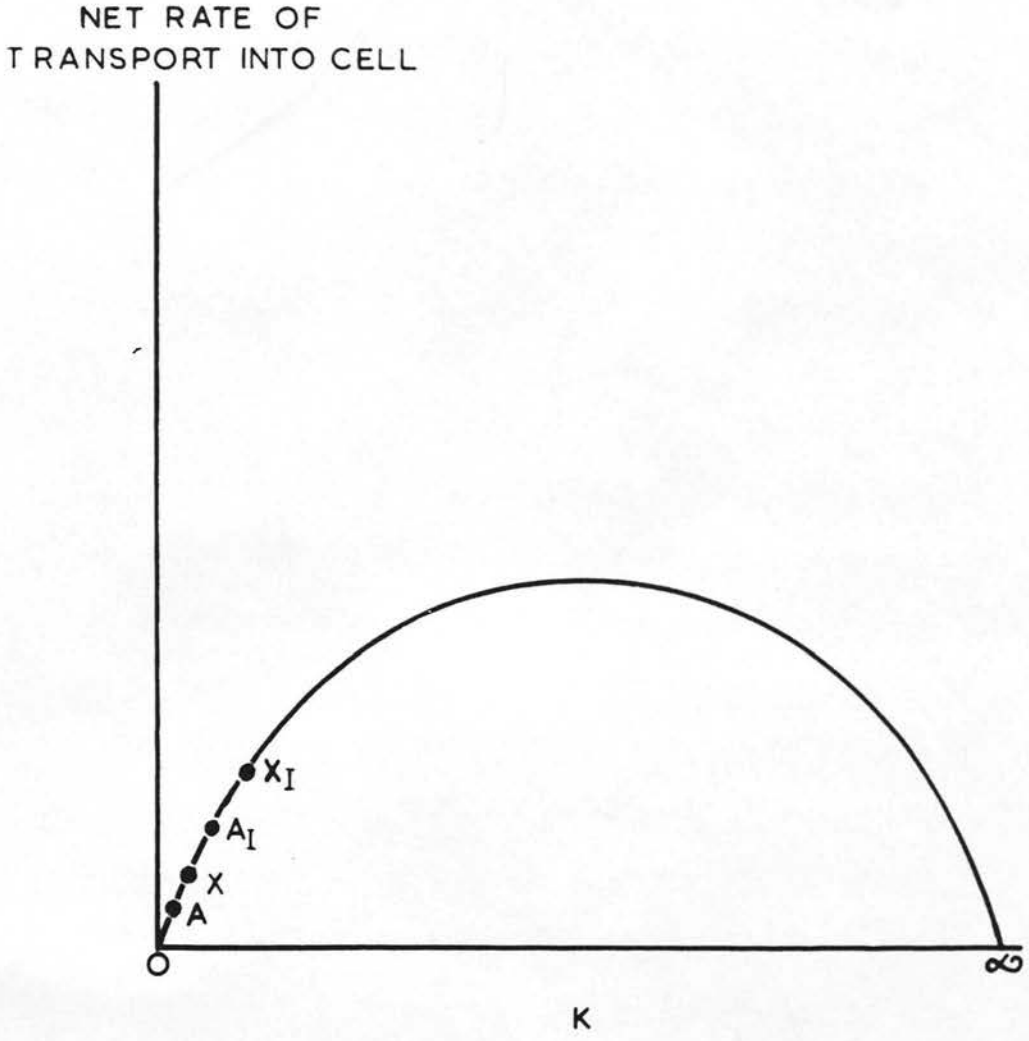
Comparison of the results for the two sugars

It is difficult to explain the basis for the difference in the rate of transport of the two pentoses by comparing the parameters. It was seen that there is good agreement between the values of V obtained for the sugars under different conditions. Since V is equal to $\frac{Ck_3}{2}$, where 'C' is the total carrier involved in transport and 'k_3' is the velocity constant for movement of the carrier across the membrane, such agreement is what one would expect. Neither 'C' nor 'k_3' would be expected to be different for the two sugars.

There is, however, a large difference in the apparent affinity of the carrier for the sugars. Whether or not insulin is present, the K value of xylose is considerably larger than that of arabinose. Yet of the two sugars, under comparable

FIG. 45.

RELATIONSHIP BETWEEN TRANSPORT RATE AND MAGNITUDE OF K



conditions, xylose penetrates the heart cells more rapidly than does arabinose. Thus the greater speed of entry is associated with the larger value of K and this is what is predicted by the permeation model because of the relationship between the transport rate and the apparent affinity of the carrier. This point was made with the use of a table in the General Introduction (p. 19) and Fig. 45 serves as an illustration here. In the absence of insulin, the K values are in the region where the transport rate is proportional to the magnitude of the parameter the xylose value (x) being larger than the arabinose value (A).

The apparent equilibrium constant has been shown to equal $\frac{k_2+k_3}{k_1}$. Assuming k_3 to be the same for both sugars, it is not possible with the data at hand to attribute the difference in the K values to a difference in the magnitude of either k_2 or k_1 or both.

The nature of insulin action

In considering possible mechanisms by which insulin exerts its effects on sugar transport, the following changes in the parameters of the trans-

port process as it is studied here have been established and must be accounted for.

(i) The addition of 0.2 mU. insulin/ml. of perfusate increases the values of K for xylose and arabinose, that is, reduces the apparent affinity of carrier for sugar. This finding confirms that of Fisher and Zachariah (1961). It is envisaged that the addition of insulin results in the shifting of the K values of the sugars from the points x and A of Fig. 45 to the points x_1 and A_1. With the addition of this submaximal concentration of insulin, there is a slight diminution in the magnitudes of V for the sugars as found by Fisher and Zachariah.

(ii) Increasing the insulin concentration above 0.2mU./ml. has very little effect upon the values of K for the sugars, thereby confirming the prediction made on the basis of the hypothesis of insulin action proposed by Fisher and Zachariah.

Where V is concerned, however, the values increase in an approximately linear fashion as the insulin concentration is increased. Thus the value of 1.82mM/min which was the average value for the two sugars in the absence of insulin, was

exceeded when the insulin concentration was raised to 1.0 mU./ml. or to any higher concentration than this. This finding is not what was predicted by the original hypothesis of Fisher and Zachariah, and demands that this hypothesis be modified.

From these findings, some speculation as to the possible sites of insulin action within the transport system can be attempted. It should not be forgotten, in this connection that the evidence suggests that the kinetics of the transport process are the same in the presence of insulin as in its absence, and hence that the same basic process is operating. We have, therefore, to consider the changes occurring in the following terms on the addition of insulin.

$$K = \frac{k_2 + k_3}{k_1} \quad \text{and} \quad V = \frac{Ck_3}{2}$$

The constants k_1 and k_2 appear only in the parameter K, so that changes in k_1, or k_2 or in both would not affect V, whereas this parameter is markedly altered by insulin.

If insulin influences permeability by altering only the amount of carrier, C, involved in transport, then K should not be altered by insulin, and this is not the case.

The simplest explanation of the effects of insulin on K and V would therefore appear to involve changes in the magnitude of the term common to both parameters, k_3, the rate constant of movement of the carrier and sugar-carrier complex across the cell membrane.

The possible implication of pinocytosis

Before considering the consequences of an increase in k_3 with respect to the carrier model of sugar transport, some attention should be paid to another possible transport mechanism, for the nature of the increase in V at higher insulin concentrations suggests that any effect on k_3 is a very marked one. If a process such as pinocytosis were involved in the transport of sugar, the addition of insulin could step-up the number of pinocytotic vesicles occurring per unit area of membrane at any time, or increase the rate of turnover of the usual number found in the absence of hormone, or both. In this connection, Paul and Pearson (1957) have observed a stimulation of pinocytosis by insulin in cultures of HeLa cells, and Barnnett and Ball (1960) have reported that insulin can induce pinocytosis in adipose tissue.

The electron micrograph prepared from a heart perfused in the absence of sugar, inulin or insulin showed that vesicles are present in the muscle cells under the conditions of our experiments (Fig. 40). However, when a direct experiment was performed studying sugar uptake in the absence of insulin, and then in the presence of two widely different insulin concentrations, it was found that there was no difference in the number of pinocytotic vesicles in the cell membranes under the different conditions,(Table 39). Experiments similar to these have been performed recently by Orth and Morgan (1963), and their results confirm the ones reported here. It should be noted, that neither of these two sets of experiments excludes the possibility that it is the rate of turnover of the vesicles of the membrane, and not simply their number, which is increased by insulin.

However, the general characteristics of sugar transport into the heart cells in the presence and absence of insulin are most readily explained by the carrier hypothesis, and it will be seen from what follows that the results of the

present work are readily explained on the basis of this hypothesis, so that there is no good reason for discarding it.

An explanation of the effects of insulin on sugar transport

From the foregoing considerations, it will be appreciated that the mechanism by which insulin accelerates sugar transport in the muscle cell could be the net result of rather complex effects upon the different components of the transport system. It is pertinent here, however, to give a simple explanation.

Let us assume that all the carrier molecules (C) of the cell membrane are involved in sugar transport in the absence of insulin, and that these carrier molecules move through the membrane at a velocity given by the transfer constant k_3 whether they are combined with sugar or not.

On the addition of 0.2 mU. insulin/ml. perfusate, let us assume that a number of the carrier molecules (c^1) bind the insulin molecules, and that the transfer constant of this insulin-carrier complex is much greater than k_3 and becomes k_3^1. In these circumstances, in spite of the

increase in the transfer constant from k_3 to k_3^1 V could decrease in magnitude, since c^1 the number of carrier molecules involved in the transport of sugar is likely to be very much smaller than C, the total number of carrier molecules in the membrane.

It should be mentioned again that in the presence of insulin, effective sugar transport will be mediated almost entirely by the insulin modified carrier molecules, so that the effective number of molecules will be c^1 (see Section I, p. 31). Where K is concerned, on the addition of insulin, $\frac{k_2+k_3}{k_1}$ will increase to $\frac{k_2+k_3^1}{k_1}$ and since this latter is the value of K for the insulin-modified carrier molecules, and these are almost entirely responsible for sugar transport, then this will be the experimentally determined value. Thus K is increased by insulin.

On adding more insulin to the system, the only effect should now be to produce more molecules of insulin modified carrier. Thus c^1 increases to c^{11}. K should not be affected by

this change, and should remain at the high value obtained in the presence of 0.2 mU insulin/ml. On the other hand, since V is a function of both the number of molecules of carrier (or insulin modified carrier) involved in effective transport and the transfer constant, then it would be expected to increase as c^{1} increases to c^{11}. Addition of yet higher insulin concentrations should again not affect K, but should increase V by increasing c^{11} to c^{111} and so on. These postulated alterations in the magnitudes of the components of the transport system are illustrated in Table 40.

An indication of the feasibility of the explanation of insulin action given above can be gained by estimating the changes in K_o and V_o observed when insulin was added. Thus the K_o value of xylose increased thirty-eight fold due to the addition of 0.2 mU. insulin/ml. while that of arabinose increases thirty-two fold. Clearly, since V_o should be affected to a greater extent that K when k_3 is increased, then when all of the membrane carrier molecules have been modified by insulin, ($c^{11111} = C$) V_o should be more than forty times higher than it was in the absence of insulin

TABLE 40

Effect of Insulin on K and V

Insulin Concentration of Perfusate(mu./ml.)	Expression For K	Expression For V
0.0	$\frac{k_2 + k_3}{k_1}$	$\frac{Ck_3}{2}$
0.2	$\frac{k_2 + k_3^1}{k_1}$	$\frac{c^1 k_3^1}{2}$
1.0	$\frac{k_2 + k_3^1}{k_1}$	$\frac{c^{11} k_3^1}{2}$
4.0	$\frac{k_2 + k_3^1}{k_1}$	$\frac{c^{111} k_3^1}{2}$
20.0	$\frac{k_2 + k_3^1}{k_1}$	$\frac{c^{1111} k_3^1}{2}$

C = Total carrier molecules of membrane

c^1, c^{11}, c^{111} and c^{1111} = Numbers of insulin modified carrier molecules at different, increasing insulin concentrations.

k_3 = Transfer constant of carrier or sugar carrier complex

k_3^1 = Transfer constant of insulin modified carrier or insulin-carrier-sugar complex.

$k_3^1 > k_3$

$C > c^{1111} > c^{111} > c^{11} > c^1$

if our explanation of insulin action is valid. In fact, at the highest concentration of insulin used (20 mU/ml.), V increases more than 100 times above its original value in the absence of insulin (from 1.82 mM/min. to 191.7 mM/min.) see Tables 37 and 38.

It is necessary to consider the significance of this explanation of insulin action at the molecular level. It is simplest to envisage the carrier as a large molecule, possibly a protein, with at least two types of binding sites. The first type can be assumed to be free to bind different sugars to different extents, and the second type could cause binding to occur between the carrier and other molecules within the membrane. There is evidence in support of this assumption. When a heart is perfused for long periods (see Section III, Fig. 25, p.144) the permeability properties fall at first as endogenous insulin activity is lost, then the permeability remains constant for over an hour, before finally increasing as the heart fails. Thus the carrier molecules associated with these sugar permeation properties must be somehow prevented from leaving the membrane. If this were not so

the permeability to sugars would be expected to fall to zero as the carrier molecules were washed out by the process of perfusion. There is ample evidence for the loss of other materials intimately associated with the functioning of the heart, in this fashion (p. 170).

The difference in the K values of xylose and arabinose should result from a difference in the degree of binding of the sugars to the sugar-binding sites, so that the true affinities ($\frac{k_2}{k_1}$) of the carrier for the sugars are different. However, due to the interaction between the second type of binding sites on the carrier molecule and sites on other membrane molecules, it is reasonable to assume that the movement of the carrier and of the sugar-carrier complex through the membrane will be hindered. The value of k_3, the transfer constant, is therefore taken to be very small.

Let us assume that when insulin is added it becomes bound to the membrane. Several groups of workers have suggested that this occurs (Stadie, Haugaard and Vaughan, 1952; Cadenas, Kaji, Park and Rasmussen, 1961; Edelman, Rosen-

thal and Schwartz, 1963). If the insulin molecules become bound to the second type of binding sites on the carrier molecule, and if free groups on the insulin molecule do not interact with membrane components, then clearly, such a combination should prevent or at least diminish the restrictions imposed upon the movement of the carrier by the other membrane components. The transfer constant of the insulin-modified carrier, k_3^1 should therefore be larger than k_3. Addition of higher concentrations of insulin should merely increase the number of carrier molecules modified by insulin and hence increase the number transporting sugar across the membrane at the faster rate, k_3^1.

General Comment

This work is of significance in relation to the effects of insulin on the carbohydrate metabolism of the animal body in as much as abundant evidence suggests that the same mechanism of sugar transport operates for the non-metabolised pentoses studied here as for the physiologically encountered sugar, glucose (Park, Reinwein, Henderson, Cadenas and Morgan, 1959; Fisher and Zachariah, 1961;). Thus the effect of insulin on accelerating glucose uptake by some tissues is likely to be mediated by the same mechanism as that by which it accelerates the uptake of xylose and arabinose by the perfused rat heart. Further than this it is not possible to homologise the results obtained in the present work with those obtained for the transport of glucose into cardiac muscle cells, since it is felt that no sound approach has yet been made to the problem of quantitatively assessing the effects of insulin on glucose transport. Park and his colleagues (Morgan, Henderson, Regen and Park, 1961; Morgan, Cadenas, Regen and Park, 1961; Post, Morgan and Park, 1961), have performed numerous experiments to this

end by studying the glucose uptake of the perfused rat heart and the glucose lost from the system by utilisation in the presence and absence of insulin. There are, however, serious objections to these experiments.

(i) Glucose transport is studied over the first 30 min. of perfusion directly after excising the heart from the animal. It has been shown in Section III of this thesis that the permeability of the heart cells to sugars is changing during this period.

(ii) The freshly excised heart contains a significant amount of endogenous insulin activity, and this is not eliminated from the heart until approximately 30 min. of perfusion have passed.

(iii) The results of Park and his colleagues indicate that over a wide range of perfusate glucose concentrations, the glucose space of the heart is smaller than the sorbitol (extracellular) space. Consequently, either glucose penetrates the extracellular space at a much slower rate than does sorbitol, and glucose has not equilibrated with this space at the end of the 30 min.

perfusion period, or the procedure used to determine the heart glucose concentration is unsound. Evidence that the former explanation is not sound is given by Morgan, Henderson, Regen and Park, (1961). As to the second explanation, it is likely that the procedure used by the workers to estimate glucose in the heart permits glycolysis to occur in the tissue after removal from the perfusion apparatus, so that free glucose is lost from the heart.

(iv) All the measurements of the glucose space made by the workers have been based on the assumption that 25% of the cell water of the hearts is inaccessible to sugar both in the presence and absence of insulin. The results of Section III of this thesis indicate that this assumption is in error.

(v) In using the sugar transport equation, Park's group determined the mean glucose uptake over the 30 min. perfusion period by measuring the difference between the initial and final concentrations of glucose in the perfusate. The perfusate glucose concentration is therefore falling throughout the experi-

ment, and since the relationship between glucose uptake and the perfusate glucose concentration is non-linear, the mean glucose uptake as measured by these workers is not related to the 30 min. perfusion time in the manner demanded by the transport equation.

Since it is not profitable, therefore, to compare the results of the present work with those of Park's group, attempts are at present being made in this laboratory to find a procedure by which the metabolic destruction of free glucose by the heart cell is prevented without impairing the functional integrity of the glucose transport system. If this can be achieved, quantitative assessment of the effects of insulin on glucose transport can be made by using similar techniques to those used here for non-metabolised pentoses.

In addition to this work, information on these problems might be gained by investigating the nature of the groups involved in the binding of insulin to the cell membrane. In past years the concept of the cell membrane has been built

up largely from observations on the phenomenon of permeability. It is to be hoped that soon our understanding of permeability will be based on a better knowledge of the morphology of the cell membrane.

CHAPTER V

Summary

1. The uptake of xylose and arabinose by the perfused heart has been studied at different time intervals up to 1 hr. in the presence and absence of insulin.

2. Over a wide range of sugar concentrations, both in the presence and absence of different insulin concentrations, the sugar uptake results conform well to the permeation equation developed in Section I and based on the carrier transport model of Widdas (1954).

3. Values for the permeation parameters, K and V have been directly determined for xylose and arabinose by plotting the sugar uptake results in a manner based on the permeation equation so that the slope and intercept of the line produced readily give the parameters by simple calculation.

4. In the absence of insulin the values of K are very small, indicating that the carrier has a very high apparent affinity for the sugars. The values of V for the sugars are in good agreement.

5. When a low submaximal concentration of insulin is added, the value of K for both sugars increases more than thirty fold and the value of V diminishes in each case. Again the values of V are in good agreement.

6. At higher insulin concentrations, the K values remain reasonably constant at the high level obtained with the addition of the lowest insulin concentration, but the values of V increase in direct proportion to the insulin concentration.

7. The simplest explanation of these changes in the permeation parameters involves a combination of insulin with the membrane carrier component in such a way that the insulin-modified carrier traverses the membrane more rapidly than does the carrier alone. This will increase the speed of sugar transport. Addition of larger quantities of insulin should then increase the number of insulin-modified carrier molecules transporting sugar at the faster speed.

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